Low-dose effects of Bisphenol A on human primary vascular endothelial cells and colon cancer cells

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GRAU DE DOUTOR EM BIOLOGIA

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LISBOA
2014

À minha filha;
aos meus pais;
ao meu marido;
aos meus amigos.

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Abstract

Bisphenol A (BPA) is an extensively utilized endocrine disruptor for which human exposure is considered generalized through ingestion. Information regarding BPA effects on vascular and digestive tract tissues is scarce. Therefore, in this work primary Human Umbilical Vein Endothelial Cells (HUVEC) and human colon adenocarcinona cell line HT29 were used to evaluate BPA effects at two distinct low-dose concentrations relevant in terms of human health risk assessment.

BPA differentially affects the cell types studied, with more pronounced aneugenic effects, nucleolar disruption and transcriptional deregulation observed in HUVEC. Prolonged BPA exposure affects aging processes in senescent HUVEC. Interaction experiments involving expression of key cancer related genes shows that BPA antagonizes transcriptional effects of the chemotherapeutic agent doxorubicin in HT29. Additionally BPA aneugenic effects are enhanced by co-exposure with *Eupatorium cannabinum* L. ethanolic extract, a medicinal plant, for which a potent cytotoxic activity against HT29 cells is also demonstrated here.

Altogether these results support increasing concerns regarding harmful effects of BPA at low-dose on human health and draw attention to the importance of a deeper understanding of BPA potential interactions with other chemicals.

K**ey-words:** Bisphenol A (BPA), HUVEC, HT29, Gene transcription, Drug interaction.

Efeitos de doses baixas de Bisfenol A em células endoteliais vasculares primárias e em células de cancro de cólon humanas

Resumo

O bisfenol A (BPA) é um desregulador endócrino amplamente utilizado para o qual a exposição humana é considerada generalizada através de ingestão. A informação sobre os efeitos do BPA nos tecidos vasculares e digestivos é no entanto reduzida. Assim, neste trabalho foram utilizadas células endoteliais da veia do cordão umbilical humanas (HUVEC) e uma linha celular de adenocarcinoma de cólon HT29 para avaliar os efeitos de duas concentrações de BPA, consideradas doses baixas e relevantes para avaliação de risco para a saúde humana.

No que respeita à indução de aneugenia, alterações nucleolares e desregulação da transcrição génica foram observados efeitos mais pronunciados do BPA em HUVEC comparativamente com HT29. A exposição prolongada ao BPA interfere com o processo de envelhecimento em células HUVEC. A avaliação de interações em HT29 revelou um efeito antagonista do BPA, na transcrição de genes associados ao cancro, relativamente ao agente quimioterapêutico doxorrubicina. Também em HT29 foi observado um aumento dos efeitos aneugénicos do BPA após exposição conjunta com extrato etanólico da planta medicinal *Eupatorium cannabinum* L., para o qual foi também demonstrada uma potente atividade citotóxica.

Os resultados obtidos suportam a crescente preocupação com os efeitos nocivos do BPA na saúde humana e acentuam importância de uma avaliação aprofundada das possíveis interações do BPA com outros compostos químicos.

Palavras-chave: Bisfenol A (BPA), HUVEC, HT29, Transcrição génica, Interação entre compostos químicos.

Preamble

Bisphenol A (BPA) is one of the greatest endocrine disruptor chemicals produced worldwide, employed in a wide variety of consumer products including food and drink containers. Human exposure to BPA through oral intake is considered generalized, at least in developed countries. However the levels of BPA internal exposure and its potential harmful outcomes to human health are still controversial. Extensive research in human cell lines, particularly from sex hormone responsive tissues, show that BPA can endorse very distinct cellular responses through estrogen receptor signalling pathways. Although BPA is absorbed in the gut and enters blood circulation, its effects on cells from digestive or vascular systems are largely unknown.

Therefore, in this research project two distinct cell types: Human Umbilical Vein Endothelial Cells (HUVEC), an *in vitro* model for vascular cells, and the human cell line HT29, originated from a colon adenocarcinona, the most common type of gastrointestinal cancer were utilized. Two BPA concentrations (44 nM and 4.4 μM), relevant in the context of risk assessment, were selected for evaluation of cellular proliferation and viability, aneugenic capacity, nucleolar organization and gene transcription in both cell types. Considering that distinct epidemiological studies correlate circulating BPA levels with age-related vascular diseases HUVEC were used to assess the effects of BPA prolonged exposure on aging cells. Furthermore there are some evidences that the action of chemotherapeutic drugs can be antagonized by BPA. Hence this work aimed also to evaluate in HT29 cells potential interactions of BPA with Doxorubicin (DOX), commonly used in chemotherapy, as well as with the extract of *Eupatorium cannabinum L.*, a plant utilized in alternative medicine for treatment of several pathologies including cancer.

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1 Introduction

Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl) propane is an organic compound and one of the greatest volume industrial chemicals utilized in the world, with a production 4.6 million tonnes in 2012 (MRC 2014). BPA was first synthesized by A. P. Dianin in 1891 and its estrogenic properties were hypothesized in the search for synthetic estrogens in the 1930s, however BPA was abandoned for pharmaceutical use when Diethylstilbestrol (DES) was found to be more efective (reviwed in Vandenberg et al. 2009). Since 1940, BPA has been extensively used in the production of a variety of polymers such as polycarbonate plastics and epoxy resins and therefore employed in the manufacture of a variety of indoor applications and consumer products (Geens et al. 2011). In the 60´s several studies emerged focused on the hypersensitivity and metabolism of BPA in different model systems (Fregert and Rorsman 1962; Gaul 1960; Knaak and Sullivan 1966) and since then extensive research has been conducted on the effects of this endocrine disrupting chemical (EDC) in both animals and humans (reviewed in Rochester 2013; Vandenberg et al. 2009). Importantly, in 1993 Krishnan and coworkers accidentally discovered that BPA leaches from autoclaved polycarbonate flasks and showed for the first time its positive effect on the proliferation rate of the human cell line MCF-7 (Krishnan et al. 1993). In 2001 the United States National Toxicology Program's Report of the Endocrine Disruptors Low-Dose Peer Review recognized that there was credible but not conclusive evidence that low doses of BPA can cause effects on specific endpoints (NTP 2001). Since 2006, the European Food Safety Authority (EFSA) conducted several scientific assessments on BPA, repeatedly concluding that there is no concern for human health (EFSA 2014). However in 2011 the European Legislation banned the use of BPA in the manufacture of baby bottles (EC 2011) and in 2012 EFSA decided to carry out a new risk assessment of BPA that is currently in progress (EFSA 2014).

1.1 Human exposure and biomonitoring

Environmental human exposure to BPA is considered a generalized phenomenon, at least in developed countries, since analysis of tissue and fluid samples reveal the presence of BPA in the majority of the individuals analysed (Geens et al. 2012). Ingestion of contaminated food is estimated to contribute with more than 90% to the overall BPA environmental exposure for all age groups and a recent intervention study showed that the removal of packed food from diet results in a significant reduction of urine BPA levels (Rudel et al. 2011). Non-dietary sources, such as air or contact, are generally considered to be significant only for occupationally exposed individuals (Geens et al. 2012). In 2004 the European Legislation established the specific migration limit (SML) for BPA on plastic materials and articles intended to come into contact with food at 0.6 mg/kg food (EC 2004). This SML value was based on an adult average body of 60 kg, the consumption 1 kg of food, and a tolerable daily intake (TDI) of 0.01 mg/kg bw (EC 2002). Although the TDI value was later raised to 0.05 mg/kg bw due to the elimination of an uncertainty factor of 5 concerning reproduction and development (EFSA 2006, 2010), the SML value was not altered. The actual TDI for BPA was derived from a comprehensive tree-generation study in rats with a No Observable Adverse Effect Level (NOAEL) of 5 mg/kg body weight/day and the application of uncertainty factor of 100 (10 for inter-species differences, 10 for inter-individual differences) (Tyl et al. 2002). This value corresponds also to BPA oral reference dosage (RfD) established by the U.S. Environmental Protection Agency (EPA) (EPA 1993) based on a rat chronic toxicity study regarding mean body weight with a Lowest Observable Adverse Effect Level (LOAEL) of 50 mg/kg body weight/day considering an uncertainty factor of 1000 (10 for inter-species differences, 10 for inter-individual differences and 10 for the uncertainty of duration of the toxic effects) (NTP 1982).

Human exposure to BPA has been estimated either by direct measurement on human biological samples or from dietary studies where concentrations in food and beverages were measured and aggregate exposure derived from food and water consumption data (reviewed in Sekizawa 2008). For the past years

urinary concentrations of total (free and conjugated) BPA have been evaluated in order to assess BPA exposure as the matrix of choice for biomonitoring studies (Calafat et al. 2008; Vandenberg et al. 2010). The most recent worldwide biomonitoring study base on data from urinary BPA concentrations estimated human exposure to be 0.27 µg/kg body weight/day for the general population, 0.78 µg/kg body weight/day for children and 0.45-1.61 µg/kg body weight/day for infants (WHO 2010). Moreover the scientific EFSA panel on "food additives, flavourings, processing aids and materials in contact with food" assumed more conservative scenarios and estimated that human exposure to BPA is in average of 1.5 µg/kg body weight/day (EFSA 2006). These results correspond to approximately 100 to 10 fold less than the TDI. Accordingly data from urinary excretion also estimate the daily intake of BPA at levels considerably lower than the TDI. Urinary excretion data analysis on US population survey NHANES 2005-2006 resulted in median intake levels of 0.032–0.036 µg/kg body weight/day with a upper level of 0.255 µg/kg body weight/day (Lakind and Naiman 2010). Identical values where also obtained through the compilation of distinct independent studies (Geens et al. 2012). However, these values are still controversial since biomonitoring data from non-urinary human samples point to higher human BPA exposure. Considerably high concentrations of BPA have been measured in human placental samples as well as in fetal serum indicating that the placenta does not work as a barrier to BPA and therefore that human developing fetuses are chronically exposed to BPA (Schonfelder et al. 2002). Accordingly, several inconsistencies have also been described between the estimate intake levels and biomonitoring studies performed in human blood/serum samples. These studies demonstrate that the long term daily intake of BPA leads to a steady-state presence of unmetabolized BPA in the range of 0.5–3 ng/ml (2–13 nM) (Vandenberg et al. 2007; 2010), which is about 10-fold higher than the worst case predictions for daily human exposure to BPA (Völkel et al. 2002). Also physiologically based model studies estimate that for adult humans these levels of unmetabolized BPA in blood would require dosages considerably higher than the TDI (Edginton and Ritter 2009; Fisher et al. 2011). Overall this suggests that either BPA intake is higher than estimated or that this chemical bioaccumulates in the body.

On the other hand, for the past years epidemiological studies have established positive correlations between urinary or blood BPA concentrations and the prevalence of recurrent human diseases. These include thyroid hormone disruption (Wang et al. 2013); reproductive malfunctions (Ehrlich et al. 2012; Li et al. 2011), type-2 diabetes mellitus (Silver et al. 2011), obesity (Trasande et al., 2012) and pathogenesis of age-related diseases such as coronary and carotid atherosclerosis (Lind and Lind 2011; Melzer et al. 2010; Melzer et al. 2012).

1.2 Metabolism and toxicokinetics

The metabolism and toxicokinetics of BPA has been studied in rodents (Doerge et al. 2010a, 2011), non-human primates (Doerge et al. 2010b; 2011) and humans (Völkel et al. 2002; 2005). After ingestion, BPA is absorbed from the gastrointestinal tract and undertakes a rapid phase II metabolism in the gut and liver. BPA is extensively conjugated with glucuronic acid to BPA-glucuronide resulting in loss of estrogenic activity and consequent low circulating levels of active (free) BPA (Hengstler et al. 2011). Although BPA glucuronidation is the primary mode of phase II metabolism in both rodents and primates there are some differences regarding excretion. In primates, including humans, blood BPA clearance occurs in the kidney and BPA-glucuronide represents the major BPA component in urine (Fisher et al. 2011; Tominaga et al. 2006). In contrast in rodents the conjugated BPA undergoes enterohepatic recirculation and fecal excretion via bile (Pottenger et al. 2000). It has been suggested that this difference can result in higher internal exposure in rodents due to longer elimination time and this argument was used to maintain the TDI level based on NOAEL obtained in rats since an uncertainty factor of 100 was considered conservative (EFSA 2008). Nevertheless there are evidences that regardless of the route of excretion the bioavailability of BPA is similar in rodents and primates (Taylor et al. 2011; Uchida et al. 2002).

Evaluation of BPA excretion kinetic profile in a limited number of human volunteers subjected to a single oral dose of deuterated BPA revealed a rapid peak and a terminal half-life of less than 6 hours (Völkel et al. 2002; 2005). This data has been interpreted as indicating a rapid and complete BPA clearance in

humans. However, large-scale biomonitoring data revealed that urine BPA levels do not decline rapidly with fasting time suggesting a longer BPA clearance (Stahlhut et al. 2009) what is also supported by the fact that in the 2002 Volkel study no significant removal of conjugated BPA was detected beyond 20 h of exposure (Teeguarden et al. 2005). The occurrence of BPA-glucuronide deconjugation by β-glucuronidases in specific organs and consequent conjugation–deconjugation cycling has been suggested as an explanation for the delayed excretion (reviewed in Ginsberg and Rice 2009; Vandenberg et al. 2009). Additionally, single oral bolus experiments may underestimate exposure to bioactive form of BPA. Steady state internal BPA concentrations in humans were predicted to occur in continuous exposure trough diet (Mielke and Gundert-Remy 2009) and in mice it was shown that BPA bioavailability for equivalent BPA dosages is lower for a single oral bolus administration than in continuous exposure through food (Sieli et al. 2011).

1.3 Mechanisms of action as an EDC

Although BPA estrogenic activity has long been acknowledged, it has been considered a weak estrogen due to the fact that its binding affinity to classical estrogen receptors α and β (ERα and ERβ) is 10,000- and 1,000-fold lower than that of endogenous estrogen estradiol (E2) for ERα and ERβ, respectively (Routledge et al. 2000). However, distinct studies have shown that BPA can promote estrogen-like effects similar or stronger than E2. BPA at a dose range of 0.1-1 nM was shown to be equally effective, or even more effective, than equimolar concentrations of E2 in suppressing adiponectin release from human adipose tissues (Hugo et al. 2008). In BG-1 ovarian cancer cells both BPA and E2 induce cellular proliferation by promoting the interaction between ERα and IGF-1R signaling pathways (Hwang et al. 2013b). BPA induction of alternative signaling pathways is as well a possible explanation for the paradigm of E2 and BPA equivalent effects. Interestingly, BPA binds to the orphan nuclear receptor estrogen-related receptor-γ (ERR-γ) (Matsushima et al. 2007) with 80-100 times higher affinity than for ERα or ERβ (Takayanagi et al. 2006; Thomas and Dong 2006). Also activation of membrane-bound variants of ERs, particularly ERα, that act outside the nucleus was been suggested as a possible BPA mode of

action (reviewed in Alonso-Magdalena et al. 2012). Additionally, cellular responses to BPA at low concentrations have been correlated with the trans-membrane estrogen receptor G protein-coupled estrogen receptor (GPR30 or GPER) for which BPA has relative high affinity corresponding to 2.8% of that of E2 (Shanle and Xu 2010; Thomas and Dong 2006). BPA induces rapid activation of ERK signaling pathway through GPR30 in breast cancer cells and cancer-associated fibroblasts (Dong et al. 2010) resulting in increased cell proliferation and migration (Pupo et al. 2012). Hence BPA can affect gene transcription trough nuclear and membrane-bound estrogen receptors. On the other hand, it is worth noting that hormones and EDCs, including BPA, responses do follow non-monotonic dose responses (NMDR) curves. In the case of BPA NMDR were demonstrated to emerge on exposed pituitary, prostate and pancreatic cultured cells, since very low doses can induce significant effects that are not detectable at higher concentrations (reviewed in Vandenberg et al. 2012).

1.4 Low doses effects on human cells

BPA concentrations (equal or greater than 100 µM) are genotoxic and result in a severe decrease of cellular proliferation and viability (Bolli et al. 2008; Kim et al. 2007). Additionally BPA in a range of 50-200 µM directly interferes with mechanisms of cell division by targeting tubulin promoting microtubular polymerization, which results in the formation of multiplolar spindles in HeLa cells (George et al. 2008). BPA genotoxic aneugenic properties characterized by micronuclei formation was also reported in human lymphoblastoid cell lines such as AHH-1 with a dose range of 54.12-162.8 µM (Johnson and Parry 2008) as well as in MCL-5 at 22-132 µM and further associated with chromosome non-disjunction for the concentration range of 22-88 µM (Parry et al. 2002). On the other hand, similar genotoxic effects have been describe for both BPA and E2 in MCF-7 cells although achieved with 1000-fold higher doses of BPA than the natural hormone (Iso et al. 2006). Also in CHO-K1 cells E2 induces chromosome aberrations and aneuploidgenic effects at 250 µM and 50 µM concentrations respectively, whereas BPA had the same effects for 400 µM and 500 µM (Tayama et al. 2008).

However, and considering the level of BPA exposure on human cells *in vivo*, the "low-dose" effects have been a focal point of numerous studies (reviewed in Sekizawa 2008; Teeguarden and Hanson-Drury 2013; Vandenberg et al. 2009; 2012). BPA "low-dose" effects were defined as biological changes occuring in the concentration range of typical human exposures or lower than the expected NOAEL level of 5000 µg/kg/day, used to establish the oral reference dosage (RfD) (NTP 2001). However a recent review demonstrated that the majority of *in vivo* and *in vitro* toxicity studies have been performed within the BPA concentration range of 0.1 nM to 10 µM (Teeguarden and Hanson-Drury 2013). *In vitro* cell systems have been widely used in the assessment of low-dose BPA effects however most studies have been directed to human cancer cell lines, particularly breast cancer revealing effects on proliferation and gene expression (reviewed in Vandenberg et al. 2012; Wetherill et al. 2007). It has been recently demonstrated that BPA have direct effects in vascular endothelial function (Andersson and Brittebo 2012) and acts as an E2 antagonist in colon cancer cells (Bolli et al. 2010). Nonetheless, the effects of environmental relevant levels of BPA in human vascular endothelial cells and digestive tract cells are mostly unknown, although these types of cells are in direct contact to BPA *in vivo*.

1.4.1 Cell proliferation and viability

Several studies that evaluate proliferation and viability effects of low BPA concentrations in human cells cultures reveal cell type specificities. While some cell types show a positive response to BPA (LaPensee et al. 2009; Ptak et al. 2011; Ricupito et al. 2009) in others no measurable effect is detected (Bolli et al. 2008; Kim et al. 2007; LaPensee et al. 2009). The divergence of the results has been correlated with distinct signaling pathways. BPA proliferative effects at concentrations as low as 1 nM, have been reported in ERα-positive human cell lines such as breast cancer MCF-7cell lines (Ricupito et al. 2009), human ductal breast epithelial tumor cell line (T47D) (LaPensee et al. 2009) and human ovarian carcinoma cell line (OVCAR-3) (Ptak et al. 2011). Accordingly, unresponsiveness to BPA was observed in ERα-negative ERβ-positive cell lines such as cervical cancer HeLa cells (Bolli *et al.*, 2008) or breast cancer MDA-MB-468 cells (Bolli et al. 2008; LaPensee et al. 2009). However, in SK-N-SH

neuroblastoma cells, also ERα-negative and ERβ-positive, increased proliferation induced by BPA (10 μM) was shown to be mediated by ERβ and associated with altered transcriptional levels of cell cycle-related genes (Zhu et al. 2009). On the other hand, in ERβ positive and ERα negative human testicular cancer cell line JKT-1, signaling through the membrane bound GPR30 receptor results in enhanced cell proliferation for BPA concentrations ranging from 1 pM to 10 μM, (Bouskine et al. 2009).

Cell proliferation can be naturally disrupted in the aging process with loss of replicative capacity which is associated with alterations in cell structure and physiology (Debacq-Chainiaux et al. 2008; Hwang et al. 2009). A similar phenotype can also be observed after cell exposure to subcytotoxic doses of stressful agents in a process described as stress-induced premature senescence (Dumont et al. 2002; Toussaint et al. 2000) characterized by cell-type dependent alterations in cellular gene expression profiles (Debacq-Chainiaux et al. 2008; Shelton et al. 1999). A recent study revealed that BPA (10-100 nM) may enhance senescence in normal human mammary epithelial cells (HMEC), associated to deregulation of cell cycle regulatory genes (Qin et al. 2012). However, despite the knowledge that humans are in continuous contact to BPA *in vivo*, its effects on cellular aging processes remain largely unknown. On the other hand, EDCs and particularly BPA exposure has been positively correlated with decreased semen quality and male infertility, for which induction of germ cell apoptosis is considered a primary contributing factor (reviewed in Lagos-Cabre and Moreno 2012). BPA-induced apoptosis was also observed in human trophoblastic cells *in vitro* for a concentration of 1 μM (Morice et al. 2011) as well as in rat ovarian cells *in vivo* after prolonged exposure (Lee et al. 2013), indicating a potential negative role in reproductive function. Conversely, exposure to BPA results in apoptosis inhibition in human breast cancer cell line MCF-7 for a concentration range of 10 nM – 10 μM (Diel et al. 2002) as well in non-malignant epithelial breast cells from high risk donors for a concentration of 100 nM (Dairkee et al. 2013).

1.4.2 Transcriptional and epigenetic effects

The effect of BPA exposure, in a low-dose concentration range on global gene expression profiles was evaluated in distinct cell lines including ER-positive and ER-negative Ishikawa endometrial cancer cells (Boehme et al. 2009; Naciff et al. 2010), breast cancer cells lines MCF-7 (Buterin et al. 2006), and T47D (Buterin et al. 2006) or human peripheral blood cells (PBMCs) (Wens et al. 2013). In general these studies demonstrate that BPA affects the transcriptional pattern of hundreds of genes involved in key cellular processes such as proliferation, division and apoptosis in a cell type and concentration dependent manner.

Furthermore, BPA-induced alterations on transcriptional patterns have been correlated to DNA methylation. The epigenetic effect of BPA was first demonstrated in mice after maternal exposure to BPA which resulted in decreased DNA methylation upstream of the *Agouti* gene what was prevented by maternal dietary supplementation with folic acid (Dolinoy et al. 2007). On human primary breast epithelial cells BPA low-dose exposure (4 nM) resulted in repressed expression and increased DNA methylation at CpG islands of *LAMP3 (*lysosomal-associated membrane protein 3) gene (Weng et al. 2010). Also BPA exposure of normal-like human breast epithelial cells (MCF-10F) was shown to alter DNA methylation patterns of several genes including those involved in apoptosis and DNA repair (Fernandez et al. 2012). Although there is significant evidence that BPA is able to influence DNA methylation patterns, the available data regarding BPA effects on histone modifications is almost inexistent (reviewed in Singh and Li 2012). Still, in human breast cancer cell line MCF-7 it was shown that BPA exposure increases the expression of the histone methyltransferase *EZH2* (enhancer of Zeste Homolog 2) and the overall level of histone H3 trimethylation at lysine 27 (H3K27me3) (Doherty et al. 2010).

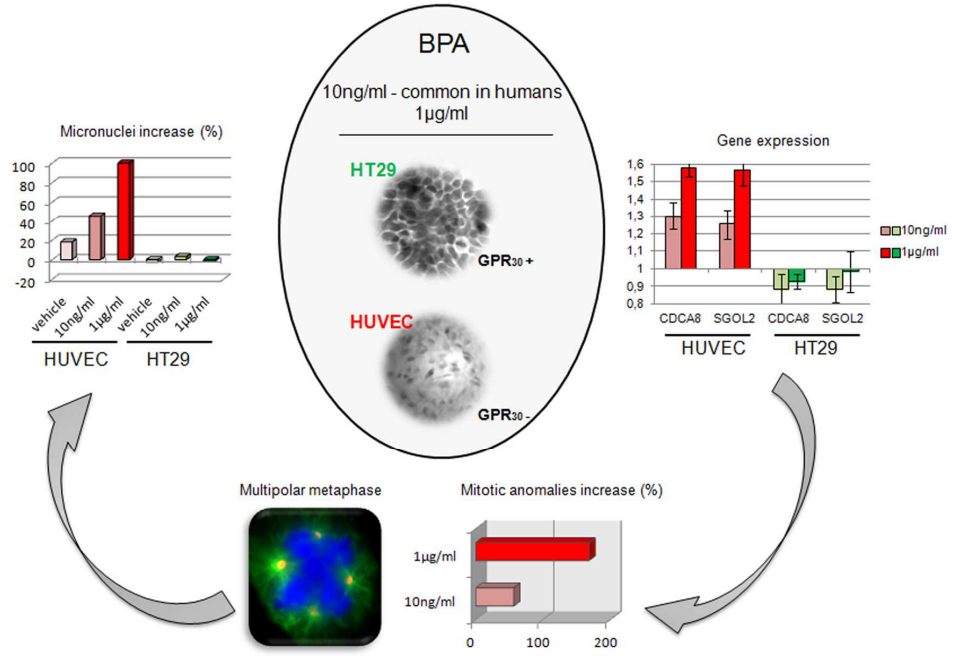
1.5 BPA interactions

In addition to the direct cellular effects induced by BPA, this chemical is also capable to interact with other compounds including natural estrogenic hormones

such as E2. In MCF-7 breast cancer cell line combined exposure to BPA at low dose and E2 at physiological concentration induces cell proliferation and decreases apoptosis (Mlynarcikova et al. 2013). Also in colon cancer cell line DLD-1 E2 pro-apoptotic action is antagonized by BPA through inhibition of cascade 3 activation (Bolli et al. 2010). Interestingly, it was also shown that BPA at environmental relevant dosages (0.01 – 10 nM) is as effective as E2 in antagonizing cytotoxicity of the chemotherapeutic agent cisplantin in breast cancer cells T47D and MDA-MB-468 (LaPensee et al. 2010). Moreover, in the same cells lines low BPA concentrations (0.1-10 nM) were also reported to antagonize the cytotoxicity of vinblastine and doxorubicin (DOX), also commonly used in cancer chemotherapy. Relevantly, in the case of BPA/DOX interaction the same study demonstrated that the BPA antagonistic effects were independent from classical estrogen receptors and potentially associated with increased expression of antiapoptotic proteins (LaPensee et al. 2009).

BPA interactions with plant-derived compounds have also been demonstrated. A few studies have suggested that some plants extracts may effectively antagonize BPA induced cytotoxicity. In hepatoma cells HepG2 two distinct medicinal plant combinations were able to antagonize the cytotoxicity of a Bisphenol-A/Atrazine mixture by restoring cellular viability up to 24-28% (Gasnier et al. 2011). In human red blood cells BPA-induced hemolysis was decreased by black tea extract or the flavonoid quercetin (Verma and Sangai 2009). Also genistein, a soy phytoestrogen was reported to efficiently suppress BPA ERα mediated proliferation trough inhibition of cell cycle progression in ovarian cancer cell line BG-1 (Hwang et al. 2013a).

2 Bisphenol A at concentrations found in human serum induces aneugenic effects in endothelial cells



Ribeiro-Varandas E, Viegas W, H Sofia Pereira, Delgado M. 2013. Bisphenol A at concentrations found in human serum induces aneugenic effects in endothelial cells. Mutation Research. 751: 27-33.

2.1 Abstract

Bisphenol A (BPA) is an endocrine disrupting chemical to which humans are exposed. Continuous environmental exposure to BPA leads to its detection in the majority of individuals from developed countries with serum concentrations ranging from 0.5 to 10 ng/ml in the general population and at much higher concentrations associated to occupational exposure. In this work, Umbilical Vascular Endothelial Cells (HUVEC) and Human Colon Adenocarcinona (HT29) cell lines were utilized to represent endothelial and digestive tract tissues which are in direct contact to BPA *in vivo*. Our results demonstrate that BPA has cell type differential effects. Relevantly, BPA concentrations commonly found in humans induce micronuclei formation and interfere in cell division processes in endothelial cells, resulting in mitotic abnormalities. We also found a BPA induced up-regulation of two genes encoding for proteins associated with chromosome segregation, namely borealin/cell division cycle A8 (CDCA8) and shugoshin-like2 (SGOL2). Taken together, the aneugenic effects observed in endothelial cells (HUVEC) substantiate increasing concerns of BPA exposure in levels currently detected in humans.

2.2 Introduction

Endocrine disrupting chemicals (EDCs) are exogenous agents that have the capacity to behave as biological signals and interfere with/or mimic estrogenic hormones. Exposure to these compounds can therefore simultaneously and differentially trigger specific signaling pathways responsible for the nature and magnitude of biological responses in diverse cell types (Shanle and Xu 2010). Bisphenol A (BPA) is a widely utilized EDC, employed in the manufacture of a variety of consumer products such as polycarbonate plastic and resins, medical tubing, toys, water pipes, and dental sealants. BPA has been detected in biological tissues and fluids of the majority of individuals in developed countries, including amniotic fluid, placenta, urine and blood. A recent extensive revision on BPA detection in humans indicates levels of internal exposure ranging from 0.5–10 ng/ml (Vandenberg et al. 2010).

Tissue specific as well as developmentally related BPA induced alterations are thought to be mediated by nuclear and/or non-nuclear estrogen receptors, which in turn are involved in various cell-signaling pathways (Welshons et al. 2006; Wetherill et al. 2007). BPA promotion of cell proliferation has been associated with nuclear estrogen receptor alpha (ERα) in distinct human cell lines (LaPensee et al. 2009; Ptak et al. 2011; Ricupito et al. 2009). On the other hand, the transmembrane estrogen receptor (GPR30) that holds much higher affinity for BPA than nuclear ER has been implicated in low dose response (Shanle and Xu 2010; Thomas and Dong 2006). BPA induced cell proliferation has been related with activation of PKA and PKG pathways via GPR30 (Bouskine et al. 2009). In a recent publication, it was also shown that BPA can induce Erk1/2/c-fos signaling through GPR30 (Dong et al. 2010).

Significantly, BPA has been characterized as an aneugenic chemical (Parry et al. 2002), and it has been suggested it directly interferes with the mechanisms of cell division (George et al. 2008). BPA induced effects include aberrations in spindle morphology, congression of chromosomes malfunctions at metaphase, nondisjunction at anaphase and abnormal microtubule organization in both cultured somatic cells and oocytes (Can et al. 2005; Lenie et al. 2008; Nakagomi et al. 2001; Pacchierotti et al. 2008; Parry et al. 2002). Furthermore, the expression of genes involved in mitotic processes also appear to be affected by BPA exposure in a variety of cell lines (Bouskine et al. 2009; Bredhult et al. 2009; Buterin et al. 2006; Naciff et al. 2010).

The aim of the present work was to investigate potential aneugenic effects of low concentration BPA exposure in two human cell types, Human Umbilical Vascular Endothelial Cells (HUVEC) and Human Colon Adenocarcinona cell line (HT29). HUVEC are primary vascular endothelial cells, and therefore models of cells that are in permanent contact with BPA *in vivo*. On the other hand, HT29 originated from the digestive tract, which is also directly exposed to BPA since exposure in humans is generally by ingestion (Vandenberg et al. 2007). The BPA concentrations used were 10 ng/ml, that is within the range detected in human blood for environmental exposure (Vandenberg et al. 2010), and 1 µg/ml within the range detected for occupational exposure (He et al. 2009; Li et al. 2010). Cytotoxic and genotoxic effects of BPA were evaluated

through analysis of cell viability, DNA integrity and micronuclei induction. Furthermore we evaluated BPA effects on mRNA levels of specific chromosome segregation related genes and performed a cytological analysis of microtubule organization and mitotic abnormalities.

2.3 Materials and methods

Cell cultures, reagents and treatments

HT29 human cell line was purchased from European Collection of Cell Cultures (ECACC,UK) and cultivated in 75cm2 flasks with RPMI media containing GlutaMAX™ I, 25 mM HEPES (Gibco), supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. HUVEC cell line was kindly offered by Dr Ana Costa from Instituto Português de Oncologia (IPO), and cultivated in 75cm2 flasks coated with 0.2% gelatin. HUVEC were grown in EGM- 2 media (LONZA # CABRCC-3162) supplemented with 5% (v/v) fetal bovine serum, 0.2% (v/v) Bovine Brain Extract (LONZA #CABRCC-4098), 0.5 ml epidermal growth factor, 0.5 ml R3 insulin-like growth factor-1, 2 ml human fibroblast growth factor, 0.5 ml endothelial growth factor, 0.5 ml ascorbic acid, 0.2 ml hydrocortisone, 0.5 ml heparin, and 0.5 ml gentamicin, for each 500 ml EGM-2. All cell cultures were maintained in a humidified 5% (v/v) CO2 atmosphere at 37°C. For treatments and experiments, HT29 cells were used between passages 2 and 15 and HUVEC between passages 3 and 7 post-confluence. BPA was freshly diluted in ethanol and added to the culture media to the final concentration of 10 ng/ml (44 nM) or 1 µg/ml (4.4 µM). After 24h cultivation, cells were incubated in medium supplemented with BPA for 24 h, 48 h or 72 h. BPA is highly stable in solution (Kang et al. 2004) and population studies have established that BPA concentrations in humans are stable over extended periods of time (Stahlhut et al. 2009). For all experiments, negative controls included cells grown in standard culture media as well as cells grown in standard culture media supplemented with a final concentration of 0.17 mM ethanol corresponding to the final concentration of ethanol in BPA treated cultures. BPA treated and control cells were analyzed simultaneously for all treatments.

Cell viability assay

Cell viability was evaluated by CellTiter-Blue assay (Promega), which is a fluorometric method that assesses cell metabolic capacity. For this, cells were plated on 96-well dishes at a density of 3.2×10^4 cells/well. Following overnight incubation, cells were treated with BPA at the specific dosages. After 24 h, 48 h and 72 h incubation, CellTiter-Blue Solution Reagent was added to each well according to manufacturer's instructions, cells incubated for 4 h and viability determined by measuring fluorescent emission at 590 nm using a Synergy HT Bio-Tek plate-reader. Experiments were repeated three times with at least three replicates per treatment.

Micronuclei analysis

Cells were cultivated on Petri dishes containing glass coverslips coated with 0.2% (v/v) gelatin (Sigma). After treatments, cells where fixed with 4% paraformaldeyde, DAPI stained and mounted on glass slides with antifade AF1 (Citifluor) mounting medium for evaluation of micronuclei formation. Images were captured using the appropriate excitation and emission filters and recorded using an epifluorescence microscope Zeiss Axioskop2 equipped with a Zeiss AxioCam MRc5 digital camera. The analysis was performed on results of at least two independent experiments with at least two replicates.

TUNEL assay

For HT29 and HUVEC cell death assessment, cells were plated on Petri dishes with 0.2% (v/v) gelatin (Sigma) coated glass coverslips. After BPA treatments, cells were fixed in 4% (v/v) paraformaldehyde for 10 min at room temperature and subsequently treated with TUNEL assay kit (in Situ Cell Death Detection Kit Fluorescein- cat # 11684795910 Roche) according to manufacturers´ instructions. Fluorescence images were recorded for each fluorochrome using the appropriate excitation and emission filters in an epifluorescence microscope Zeiss Axioskop2 equipped with a Zeiss AxioCam MRc5 digital camera. Analysis was performed on two independent experiments with two replicates per treatment.

Immunofluorescence

Cells were plated on Petri dishes containing glass coverslips coated with 0.2% (v/v) gelatin (Sigma). After BPA treatments cells were fixed in 4% (v/v) paraformaldehyde for 10 min at room temperature and permeabilized with 0.25% (v/v) Triton X-100 for 15 min. Fixed cells were then incubated in 5% (w/v) BSA/PBS solution for 60 min. For immunodetection cells were incubated independently with the primary antidodies anti-acetyl-histone H3 (Lys 56) (ab76307, Abcam) or anti- GPR30 (ab39742, Abcam) or simultaneously with anti-αTubulin (T9026, Sigma) and anti-λTubilin (T3559, Sigma). Antibodies were diluted 1:200 in 1% (w/v) BSA/PBS and incubation was carried out at 37ºC for 1:30 h. After washing with PBS, conjugated anti-rabbit-FICT IgG (1:200, Abcam) or anti-mouse-Cy3 IgG (1:200, Sigma) secondary antibodies were added at 1% (w/v) BSA/PBS and incubated for 60 min at 37°C. Cells were then washed three times with PBS, and DAPI stained, coverslips were mounted on glass slides with antifade AF1 (Citifluor). Immunofluorescence was recorded using an epifluorescence microscope Zeiss Axioskop2 equipped with a Zeiss AxioCam MRc5 digital camera. Images were captured for each fluorochrome using the appropriate excitation and emission filters and merged with Adobe Photoshop 7.0 (Adobe Systems) software. Two replicates for per treatment were analyzed for two independent experiments.

Protein extraction and western blotting analysis

Cells were washed once with PBS and collected by trypsinization and centrifugation. For total protein lysate, pellets were resuspended in 500 µl SDS buffer (0.125 M Tris-HCL, 10% 2-mercaptoethanol, 2% SDS and 10% Sucrose) (Dong et al. 2010) and sonicated on ice 2 x 15 sec 20%. After centrifugation (14000 *g*), the supernatant was transferred to a fresh centrifuge tube and stored at -20ºC. Protein concentrations were determined through the Bradford method (Protein assay cat. # 500-0006 BioRad). Western blotting electrophoresis on polyacrylamide gel were performed as described in (Laemmli 1970) using 50 µg of protein samples transferred on to PVDF membranes and stained by Ponceau S reagent. The immunoblots were blocked with 3% (w/v) dry milk in PBST (0.05% (v/v) Tween 20, 137 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7

mM KCl) and incubated with primary antibodies: anti-acetyl-histone H3 (Lys 56) (ab76307), anti- GPR30 (ab39742), anti-ERb (ab3577) (dilution 1:1000, 1:400, 1:1500 respectively, Abcam) or anti-αTubulin (T9026) (dilution 1:2000, Sigma) Peroxidase-conjugated anti-rabbit (cat # 32460 Pierce Biotechnology) and anti-mouse (cat # 32430 Pierce Biotechnology) antibodies were used at dilutions 1:1250. Detection was performed with SuperSignal West Femto Maximum Sensitivity Substrate (cat # 34094 Thermo Scientific) according to the manufacturer's instructions. Immunoreactive protein bands were detected by BioRad Chemidoc XRS. Intensity levels of immunoreactive protein bands were analyzed by ImageJ software (http://rsbweb.nih.gov/ij/). Two independent protein extractions for each treatment were performed and at least three western blots were analyzed.

cDNA isolation and real-time quantitative PCR

Transcriptional analysis of cell division genes CDCA8, SGOL2, Aurora A, γ-tubulin and the control gene GAPDH was performed by quantitative real-time PCR with gene specific primers as listed in table 1. Total RNA was extracted from HT29 and HUVEC cells exposed to both concentrations of BPA for 24 h and 72 h as well as non-treated and control culture to which ethanol alone was added to the culture media. Cells were collected at 80% confluence by trypsinization, washed in PBS, centrifuged at 1000 *g*, and RNA isolated with the RNAqueous Kit (Cat # AM1912 Thermo Scientific) following manufactures´ instructions. After verifying concentration and integrity, 3 µg of total RNA was utilized for RNase free DNase digestion (RQ1 RNase free DNase, cat # M6101 Promega) and first strand cDNA synthesis was completed with random primers following manufacturers´ instructions (DYNAmo cDNA syntesis Kit, ca t# F-470L Thermo Scientific). The resulting cDNA was utilized for qRT-PCR with the BIO-RAD SsoFast Eva Green Supermix (BIO-RAD Cat # 172-5201) utilizing the following conditions; 95 °C-3 min, 35 cycles (95 °C-30 sec, 55 °C-30 sec, 72 °C-40 sec), and 72 °C-5 min. To ensure that genomic DNA was completely absent prior to cDNA synthesis, PCRs were performed with 18S primers and 250 ng of DNase digested RNA. Control PCRs were also performed for both primer combinations without template. Denaturation curves were calculated by

measuring single stranded product at 0.5°C intervals from 55°C to 95°C. After denaturation curves were observed to ensure correct amplification products, threshold cycles (C_t) were equilibrated with mean GAPDH to calculate ΔC_t (ΔC_t = C_t of interest – mean GAPDH C_t). Cell cycle associated genes expression levels were analyzed by calculating $\Delta\Delta C_t$ ($\Delta\Delta C_t$ = ΔC_t **a** – mean ΔC_t **b**, where **a** and **b** are being compared), which in turn was used to determine mean fold change ($2^{-\Delta\Delta Ct}$) ± standard deviation between treatments. Experiments were repeated three times with at least three replicates per cell treatment/primer combination in each experiment.

Table 1 Primers used for qRT-PCR.

Gene	Forward primer (5´→3´)	Reverse primer (5´→3´)
CDCA8	AAGGTAATACAGGTAGATGAA	GTTCTCTTCTTGGATGGA
SGOL2	TACATTCACCTAACATACAA	GCTCATCATCACTTACTT
AURKA	GCTGGAGAGCTTAAAATTGCAG	TTTTGTAGGTCTCTTGGTATGTG
γ-Tubulin	CTCAAGAGGCTGACGCAGAAT	CTGGCTGACATGATGGTAGACAC
GAPDH	GAGTCAACGGATTTGGTCGTA	GCAGAGATGATGACCCTTTTG

[a] GenBank accession numbers (National Center for Biotechnology).

Statistical analysis

Statistic analysis of was performed comparing cells grown in standard medium (control) to cells exposed to vehicle EtOH or BPA using Student's t Test.

2.4 Results and discussion

2.4.1 Low BPA concentrations do not affect cell proliferation or viability independently of GPR30.

Potential cytotoxic and genotoxic effects of BPA exposure were evaluated by analyzing cell viability and proliferation in HT29 and HUVEC cells using two BPA concentrations 10 ng/ml (44 nM) and 1 µg/ml (4.4 µM). The CellTiter-Blue assay was utilized to measure viability at three time periods; two corresponding to non-confluent cultures (24 h and 48 h) and one where the culture is at 80% confluence (72 h) (Figure 1). Our results show no significant differences in viability or proliferation associated with BPA exposure. Previous studies have demonstrated that high BPA concentrations (equal or greater than 100 µM) are

genotoxic, and resulted in decreased cellular proliferation as well as viability (Bolli et al. 2008; Kim et al. 2007). However, the literature shows that the effects of exposure to low BPA concentrations (in the range of 1 nM to 10 μM) are variable. Whereas no detectable effects have been reported in some cell types (Bolli et al. 2008; Kim et al. 2007; LaPensee et al. 2009), an increase in cell viability and proliferation was observed in others (LaPensee et al. 2009; Ptak et al. 2011; Ricupito et al. 2009). This discrepancy in the effects of BPA may be dependent on cell type and expression of estrogen receptors, as discussed in detail below.

Positive effects of BPA concentration as low as 1 nM on cell proliferation have been described in estrogen receptor alpha (ERα) positive human cell lines MCF-7, T47D and OVCAR-3 (LaPensee et al. 2009; Ptak et al. 2011; Ricupito et al. 2009). In fact, the lack of responsiveness in HeLa and MDA-MB-46 has been correlated with the absence of this receptor in these human cell lines (Bolli et al. 2008; LaPensee et al. 2009). Both HT29 and HUVEC cells used in this study express ERβ but not ERα (Campbell-Thompson et al. 2001; Toth et al. 2008), which could explain the lack of BPA effects on cell proliferation. However, increased proliferation was observed in ERβ positive and ERα negative cell line JKT-1 when exposed to BPA concentrations ranging from 1 pM to 10 μM (Bouskine et al. 2009). This study showed that BPA effects in JKT1 cells are mediated through a G protein-coupled receptor 30 (GPR30). Interestingly, this membrane-bound estrogen receptor has a higher affinity for BPA than ERα or ERβ (Thomas and Dong 2006) and has been proposed as a mediator of BPA effects at low concentrations (Bouskine et al. 2009; Shanle and Xu 2010; Thomas and Dong 2006). In relation to the cells used here, the expression of GPR30 had not been analyzed in HT29 and is only expressed in HUVEC cultured in specific conditions (Takada et al. 1997).

Western immunoblotting was utilized to test GPR30 expression in HT29 and HUVEC in the culture conditions utilized in this study. The expression of ERβ was confirmed for both cell lines as positive controls, evident as a specific immunoreactive protein band at 55 kDa (Figure 2A). As expected, GPR30 expression is not detected in HUVEC (Figure 2A), in agreement with pervious results showing that these cells express GPR30 exclusively under stream flow

conditions (Takada et al. 1997). Conversely, a clear GPR30 immunoreactive protein band with the expected size of approximately 55 kDa is detected in HT29 cells (Figure 2A). Immunocytofluorescence shows that GPR30 was restricted to the cytoplasm in HT29 cells (Figure 2B), with identical distribution to that previously observed in MDA-MB231 and HEC50 cells and characteristic of its endoplasmic reticulum association (Otto et al. 2008). To the best of our knowledge, this is the first time that the expression of GPR30 is demonstrated in this cell line. More importantly, the presence of GPR30 does not result in a positive response in cell proliferation in HT29 cells, in contrast with the results obtained in JKT-1 cells (Bouskine et al. 2009). This indicates that although GPR30 may have a role in mediating the responses to BPA exposure in some cell types, its expression does not result in induced cell proliferation in all GPR30 positive cells.

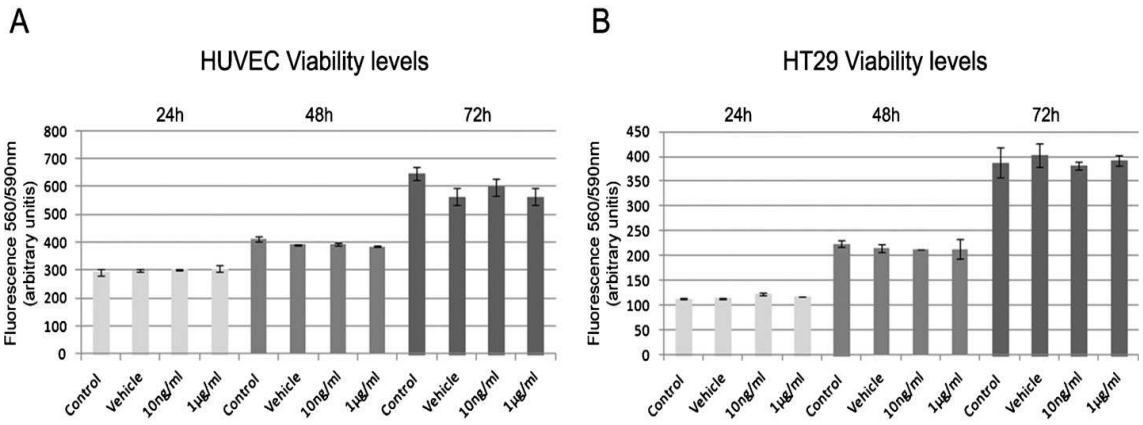


Figure 1 - BPA does not affect cell viability. Viability assay of HUVEC (**A**) and HT29 (**B**) cells after 24 h, 48 h and 72 h culture in control (control medium), vehicle (control media supplemented with 0.17 mM ethanol) and 10 ng/ml or 1 μg/ml of BPA. Cultivation and viability assays were performed simultaneously for all growth conditions. Results are presented as mean ± standard deviation for fluorescence intensity at 590 nm (using 560 nm for excitation).

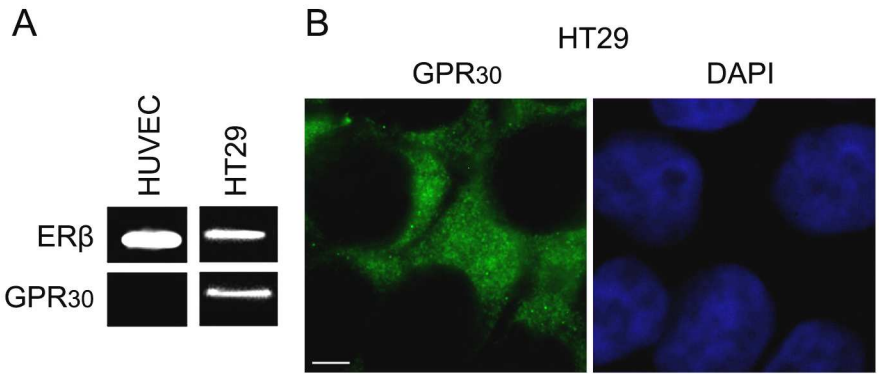


Figure 2 - GPR30 is present in HT29 cell line. (A) Western immunoblot confirmed the presence of Estrogen Receptor beta (ERβ) in both HUVEC and HT29 cell lines (upper panel). GPR30 receptor is detected in HT29 cells but not in HUVEC (lower panel). The detection of both estrogen receptors where performed on the same PVDF membrane. (B) Immunocytofluorescence images of representative interphase HT29 cells showing GPR30 distribution signals (left) and corresponding DNA DAPI staining (right), bar = 5 µm.

2.4.2 Low BPA concentrations induce micronuclei formation without affecting DNA integrity.

Potential cytotoxic and genotoxic effects of BPA exposure were evaluated by analyzing DNA integrity and micronuclei induction. Effects of BPA exposure on DNA stability and damage repair were evaluated through TUNEL assay as well as analysis of histone H3 acetylated on lysine 56 (H3K56ac). This post-translation modification of H3 was recently described to be crucial for genomic stability (Yuan et al. 2009), increasing in response to DNA damage and repair (Das et al. 2009). The TUNEL assay did not detect DNA double strand breaks or apoptotic cells associated to BPA exposure (Figure 3A). There was also no detectable variation in nuclear pattern of H3K56ac, where a dispersed distribution is observed throughout the nucleus and nucleoli regardless of cell line or BPA treatments (Figure 3B). Accordingly, western immunoblotting revealed an identical low intensity H3K56ac immunoreactive protein band with the expected size of approximately 17 kDa in all conditions tested (Figure 3C). These results indicate that the BPA concentrations utilized in this work do not affect DNA integrity or the expression of histone H3 acetylated on lysine 56 in HUVEC or HT29 cells.

Micronucleus (MT) formation occurs due to chromosome segregation errors and/or chromosome fragmentation, and is therefore a relevant assay for genotoxicity. In this context, we evaluated the frequency of micronucleated cells in the distinct growth conditions, as demonstrated in Figure 4. Our results show marked differences in sensitivity to BPA between the two cell lines. Although a high level of micronucleated cells is observed in HT29 cultured in control conditions (4.45%), there is no alteration in the percentage of these cells with micronuclei after BPA exposure (4.6% for 10 ng/ml; 4.4% for 1 µg/ml). In contrast, micronucleated HUVEC cells are significantly less frequent in control conditions (1.1%), but both BPA concentrations resulted in a significant increase in the proportion of micronucleated cells (1.6% for 10 ng/ml, t-test, $p = 0.0045$; 2.2% for 1 µg/ml, t-test, $p = 0.0002$) (Figure 4).

BPA has been described as a genotoxic aneugenic chemical capable of inducing aneuploidy and resulting in centromere positive micronuclei (Kabil et al. 2008; Parry et al. 2002; Pfeiffer et al. 1997) in a dose dependent manner. However, the concentrations for which effects are detectable vary depending on cell type. In human cells lines AHH-1 (Johnson and Parry 2008) and MCL-5 (Parry et al. 2002) induction of micronuclei was observed only for concentrations at least ten times greater than the highest concentration utilized in this work, while for MCF-7 cell line a significant increase in the proportion of micronucleated cells was observed at a concentration equivalent to the higher concentration used here (Kabil et al. 2008). Our results with HT29 and HUVEC cell lines support previously described variation in BPA induced micronuclei formation between cell types. More importantly, we show for the first time that exposure to BPA at low concentrations increase the proportion of micronuclei in the HUVEC primary cell line, establishing the potential genotoxic effects of exposure to BPA.

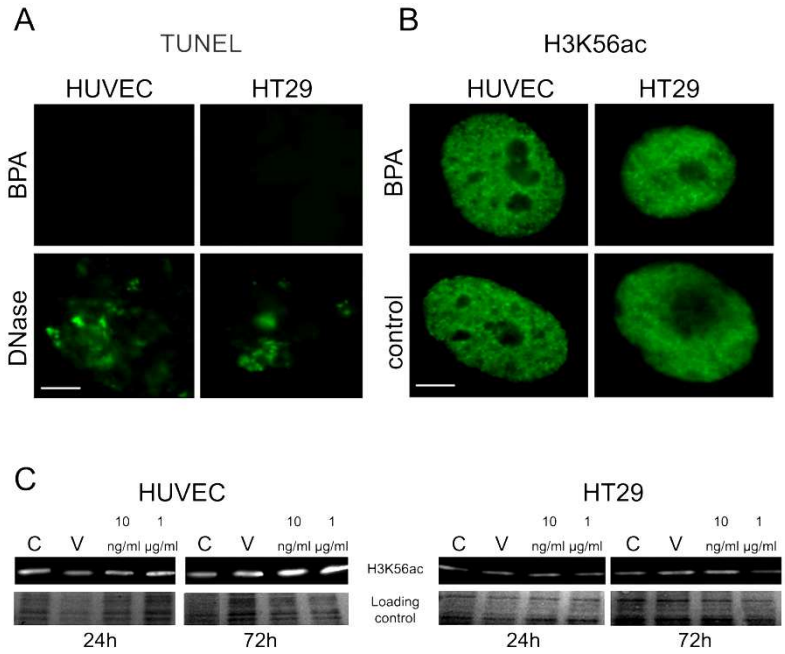


Figure 3 - BPA does not induce DNA double strand breaks or alter H3K56ac patterns. (A) TUNEL assay of HUVEC (left) and HT29 (right) cells. Cells exposed to 1 µg/ml BPA show no detectable DNA double strand breaks. Positive controls with DNase treated cells are shown on bottom. (B) HUVEC (left) and HT29 (right) cells after immunocytofluorescence detection of H3K56ac. 1 µg/ml BPA treated cells (top) show H3K56ac interphase pattern identical to control (bottom). Bars = 5 µm. (C) Immunoblotting with H3K56ac on HUVEC and HT29 cells grown for 24 h and 72 h in control media (C), vehicle (V), 10 ng/ml or 1 µg/ml BPA. An identical histone specific band corresponding to approximately 17 kDa is detected in all growth conditions. PVDF membranes stained by Ponceau S reagent before immunoblotting of total proteins between 40 kDa and 25 kDa of are shown as loading controls.

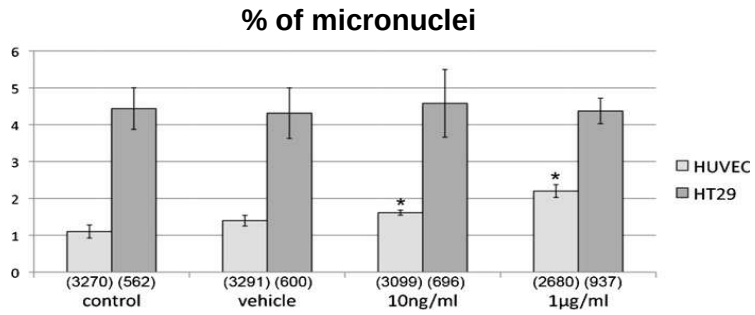


Figure 4 - BPA induces micronuclei in HUVEC cells. Percentage of micronuclei in HUVEC and HT29 cells after 72 h of culture in control, vehicle, 10 ng/ml or 1 µg/ml of BPA. The results are presented as mean ± standard deviation and total number of cells analysed is show in brackets. * a significant difference compared with the treatment with vehicle (Student's t Test, $p < 0.005$).

2.4.3 BPA differentially affects genes related with chromosome segregation.

The expression of genes encoding for proteins involved in chromosome segregation was assayed in order to provide more insight into the differential effect of low BPA concentrations on micronuclei induction in HUVEC and HT29 cells. For this purpose, quantitative RealTime-PCR (qRT-PCR) was utilized with primers specific for γ-tubulin and Aurora A centrosome components as well as two genes involved in chromatid segregation, namely CDCA8 and SGOL2. γ-tubulin is essential for microtubule polymerization (Raynaud-Messina and Merdes 2007) and Aurora A is a mitotic key regulatory protein essential for centrosome maturation, spindle organization and chromosome segregation (Anand et al. 2003; Kollareddy et al. 2008). CDCA8, also known as borealin/cell division cycle A8, is a component of the chromosomal passenger complex (CPC) and is one of the master regulators of events involved in mitotic bipolar spindle stability (Gassmann et al. 2004). Shugoshin-like2 (SGOL2) is essential for accurate chromosome segregation, involved in the regulation of the CPC and the phosphorylation of a microtubule depolymerase associated to chromosome congression and resolution of improper microtubule attachments. In fact SGOL2 depletion induces precocious dissociation of centromeric cohesion resulting in separation of sister chromatids (Illingworth et al. 2010; Kitajima et al. 2006; Tanno et al. 2010) and is fundamental for the bi-polar attachment of chromosomes (Rivera et al. 2012). Comparative gene expression values between cells exposed to BPA in vehicle and vehicle alone (mean fold change ± standard deviation) show subtle yet significant differences between BPA doses, times of exposure and treatments for two of the four genes analyzed (Figure 5). Interestingly, the expression of the two genes encoding for essential centrosome components (γ-tubulin and Aurora A) do not vary in BPA exposed HUVEC cells, in contrast to CDCA8 and SGOL2 genes which encode proteins that associate directly with chromosomes.

qRT-PCR results indicate increased CDCA8 mRNA in HUVEC and HT29 cells after exposure to high BPA concentration for 24 h (1.477 ± 0.045 and 1.333 ± 0.042 for HUVEC and HT29, respectively). After 72 h exposure, this effect is no longer observed in HT29 and maintained in HUVEC (1.577 ± 0.112). After exposure to low BPA concentrations, CDCA8 expression is altered exclusively

in HUVEC cells exposed to BPA for 72 hours (1.304 ± 0.167). Up-regulation of SGOL2 is observed solely in HUVEC cells exposed to high BPA concentration independent of the exposure time (1.431 ± 0.089 and 1.566 ± 0.116 at 24 h and 72 h, respectively) (Figure 5A). Our results of BPA induced CDCA8 and SGOL2 up-regulation diverge from previously published data showing down-regulation of these genes in HEECs cultures exposed to 50 µM BPA (Bredhult et al. 2009). This may be due to the BPA concentrations assayed, which are much lower (ranging 10 and 1000 times lower concentration) in this study and/or to cell type specificity. In fact, the present results show that different cell types have distinct sensitivities to BPA.

Our data shows that BPA has more pronounced effects on gene transcription in HUVEC than in HT29 cells. Since HT29 expresses GPR30 and in our culture conditions HUVEC do not, the observed BPA induced alterations in HUVEC cells are independent of the presence of GPR30 receptor. Moreover, we can conclude that the observed cell specific effects are not mediated by classical nuclear ERs alone, since ERα is not expressed in either cell type and ERβ is expressed in both. Taken together, quantitative Real Time PCR data establishes that low BPA concentrations (equivalent to those found in human serum) can have effects on the expression of genes involved in basic cellular processes, such as chromosome segregation.

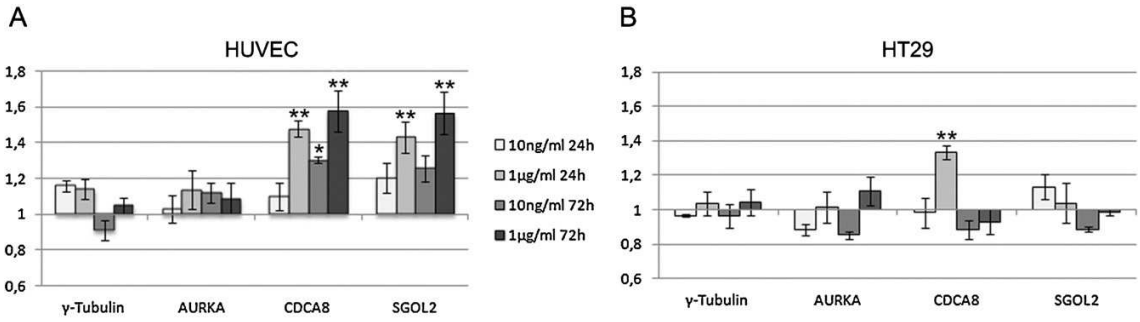


Figure 5 - BPA effects on expression of genes involved in chromosome segregation. Graphic representation showing quantitative real-time PCR analysis of γ-tubulin, AURKA, CDCA8 and SGOL2 gene transcription after exposure to BPA. Fold changes ($2^{-\Delta\Delta Ct}$ ± standard deviation) in gene expression after 24 h and 72 h of BPA exposure at 10 ng/ml or 1 µg/ml are shown for (A) HUVEC, and (B) HT29. GAPDH was utilized as a reference gene and Student's t Test significant differences in average mean fold changes are shown as **$p < 0.01$ and *$p < 0.05$.

2.4.4 Low concentrations of BPA disrupt mitotic stability and organization in HUVEC.

It was recently demonstrated that BPA concentrations at least 1000 fold higher than the lower dosage used here (10 ng/ml) directly targets tubulin, promoting microtubular polymerization and the induction of multiplolar spindles in HeLa cells (George et al. 2008). Ectopic poles were found to enclose γ-tubulin but not centrin, indicating that disruption of mitotic spindle occurs without centrosome amplification (George et al. 2008). Accordingly, it has been previously shown that higher concentrations of BPA promote multipolar spindles (George et al. 2008; Johnson and Parry 2008; Kabil et al. 2008; Ochi 1999; Parry et al. 2002) and CREST-positive micronuclei indicating the presence of kinetochores (Kabil et al. 2008; Parry et al. 2002; Pfeiffer et al. 1997). Since micronuclei and qRT-PCR analysis revealed that HUVEC cells are more sensitive to BPA than HT29, we performed cytological analysis of BPA induced alterations in cytoskeleton organization and γ-tubulin distribution throughout the cell cycle on HUVEC cells. Regarding the effects of BPA on the cytoskeleton, γ- and α-tubulin immuno labelling patterns in interphase nuclei were identical between control, vehicle and BPA treated cells. However, several aberrations were observed associated with BPA exposure in mitotic cells, including multipolar spindles with 1 or 2 ectopic poles that enclose γ-tubulin, microtubule misalignment, and absence of midbody structure at telophase cells (Figure 6A). No significant variation was found between control and vehicle (t-test, $p = 0.1287$ for 24 h and t-test, $p = 0.2166$ for 72 h, respectively). However significant increases in mitotic abnormalities in relation to control were observed for both BPA concentrations, particularly evident after 72 h of exposure (24 h: t-test, $p = 0.0087$ and t-test, $p = 0.0064$ for 10 ng/ml and 1 μg/ml respectively; 72 h: t-test, $p = 0.0078$ and t-test, $p = 0.0002$ for 10 ng/ml and 1 μg/ml respectively) (Figure 6B). Similarly to the results of BPA induced micronuclei, the effects on mitotic cells are more severe after 72 h of exposure to the higher BPA concentration (Figure 6B). The observed spindle irregularities, which have the potential to induce chromosome segregation errors and aneuploidy during mitosis, may be responsible for the increase in BPA induced micronuclei. This hypothesis is further supported by the results of the TUNEL assay as well as analysis of histone H3 acetylated on lysine 56 (H3K56ac) indicating lack of evidence of DNA fragmentation.

Interestingly, mitotic indexes revealed no differences between BPA treated and untreated cells. This is evident as average mitotic indexes of 4.5% at 24 h for all treatments, which reduces to approximately 2.5% at 72 h as expected due to cell confluence. Although cytostatic assays involving measuring mitotic indexes after removing BPA were not performed, our mitotic index and proliferation results suggest that these BPA concentrations do not have cytostatic effects on HUVEC cells.

Taken together, our results show that the low BPA concentrations found in human samples induce spindle disruption in HUVECs with formation of additional γ-tubulin positive poles. As far as we know, this is the first time that this is observed after exposure to BPA at such low concentrations.

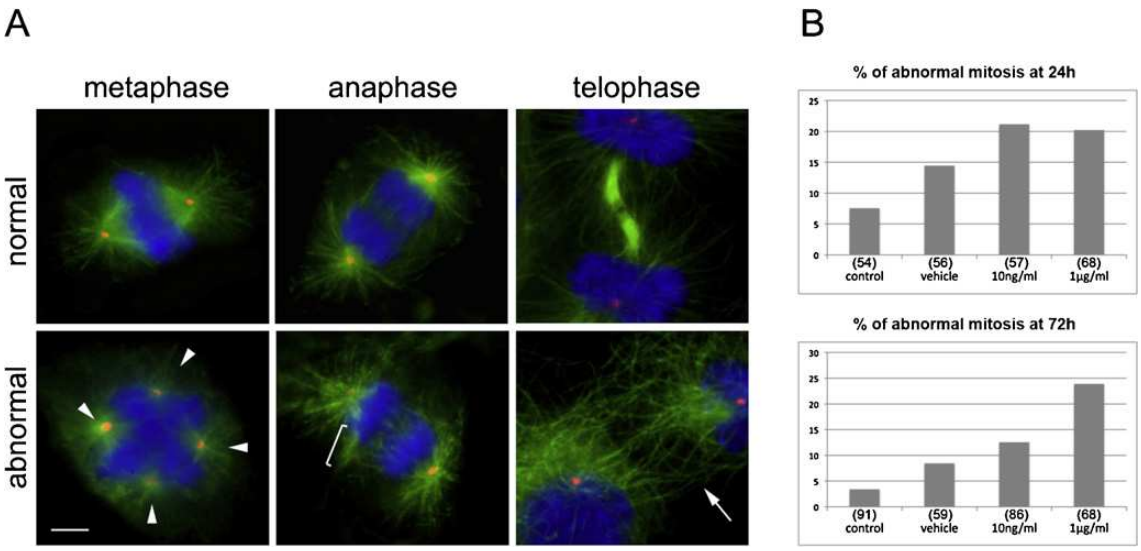


Figure 6 - BPA disrupts mitosis in HUVEC cells. (A) Immunofluorescence results of α-tubulin (green) and γ-tubulin (red) showing normal metaphase, anaphase and telophase cells (top panels). BPA induced mitotic abnormalities of cells in the same mitotic stages are shown on bottom panels: multipolar metaphase with four mitotic poles positive for γ-tubulin (arrowheads), anaphase with misaligned microtubules (bracket) and telophase lacking microtubule midbody structure (arrow). Chromosomes are DAPI stained (blue), bar = 5µm. (B) Percentage of mitotic abnormalities after 24 h and 72 h culture in control, vehicle, 10 ng/ml or 1 µg/ml BPA. The total number of mitotic cell scored is shown in brackets.

2.5 Conclusion

The effects of BPA have been extensively studied in recent years, establishing that this EDC has diverse effects on human cells that are highly variable depending on cell type. In the present study, primary cell line HUVEC is more sensitive to BPA than HT29, although both cell types are negative for ERα and positive for ERβ and only HT29 expresses GPR30. More importantly, we reveal for the first time that exposure to BPA at concentrations currently detected in humans are capable of interfering with cell division processes and result in mitotic aberrations. Our results confirm increasing concerns that BPA may have adverse effects even at extremely low concentrations (Sekizawa 2008; Vandenberg et al. 2009; Welshons et al. 2006). Studies such as this are critical for BPA risk assessment, especially considering the generalized environmental exposure of humans to this EDC.

2.6 References

Anand S, Penrhyn-Lowe S, Venkitaraman AR. 2003. Aurora-a amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to taxol. Cancer Cell 3:51-62.

Bolli A, Galluzzo P, Ascenzi P, Del Pozzo G, Manco I, Vietri MT, et al. 2008. Laccase treatment impairs bisphenol a-induced cancer cell proliferation affecting estrogen receptor alpha-dependent rapid signals. IUBMB Life 60:843-852.

Bouskine A, Nebout M, Brucker-Davis F, Benahmed M, Fenichel P. 2009. Low doses of bisphenol a promote human seminoma cell proliferation by activating pka and pkg via a membrane g-protein-coupled estrogen receptor. Environ Health Perspect 117:1053-1058.

Bredhult C, Sahlin L, Olovsson M. 2009. Gene expression analysis of human endometrial endothelial cells exposed to bisphenol a. Reprod Toxicol 28:18-25.

Buterin T, Koch C, Naegeli H. 2006. Convergent transcriptional profiles induced by endogenous estrogen and distinct xenoestrogens in breast cancer cells. Carcinogenesis 27:1567-1578.

Campbell-Thompson M, Lynch IJ, Bhardwaj B. 2001. Expression of estrogen receptor (er) subtypes and er beta isoforms in colon cancer. Cancer Research 61:632-640.

Can A, Semiz O, Cinar O. 2005. Bisphenol-a induces cell cycle delay and alters centrosome and spindle microtubular organization in oocytes during meiosis. Mol Hum Reprod 11:389-396.

Das C, Lucia MS, Hansen KC, Tyler JK. 2009. Cbp/p300-mediated acetylation of histone h3 on lysine 56. Nature 459:113-U123.

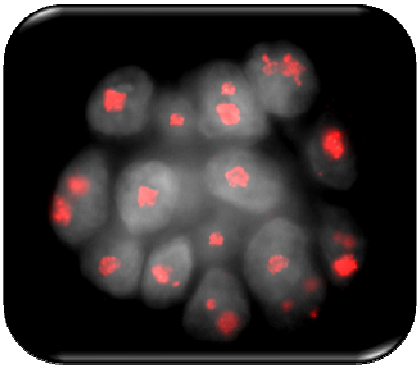
Dong S, Terasaka S, Kiyama R. 2010. Bisphenol a induces a rapid activation of erk1/2 through gpr30 in human breast cancer cells. Environ Pollut 159:212-218.

Gassmann R, Carvalho A, Henzing AJ, Ruchaud S, Hudson DF, Honda R, et al. 2004. Borealin: A novel chromosomal passenger required for stability of the bipolar mitotic spindle. J Cell Biol 166:179-191.

George O, Bryant BK, Chinnasamy R, Corona C, Arterburn JB, Shuster CB. 2008. Bisphenol a directly targets tubulin to disrupt spindle organization in embryonic and somatic cells. ACS Chem Biol 3:167-179.
He Y, Miao M, Wu C, Yuan W, Gao E, Zhou Z, et al. 2009. Occupational exposure levels of bisphenol a among chinese workers. J Occup Health 51:432-436.
Illingworth C, Pirmadjid N, Serhal P, Howe K, Fitzharris G. 2010. Mcak regulates chromosome alignment but is not necessary for preventing aneuploidy in mouse oocyte meiosis i. Development 137:2133-2138.
Johnson GE, Parry EM. 2008. Mechanistic investigations of low dose exposures to the genotoxic compounds bisphenol-a and rotenone. Mutat Res 651:56-63.
Kabil A, Silva E, Kortenkamp A. 2008. Estrogens and genomic instability in human breast cancer cells--involvement of src/raf/erk signaling in micronucleus formation by estrogenic chemicals. Carcinogenesis 29:1862-1868.
Kang JH, Ri N, Kondo F. 2004. Streptomyces sp. Strain isolated from river water has high bisphenol a degradability. Lett Appl Microbiol 39:178-180.
Kastl L, Brown I, Schofield AC. 2009. Effects of decitabine on the expression of selected endogenous control genes in human breast cancer cells. Mol Cell Probes 24:87-92.
Kim K, Son TG, Kim SJ, Kim HS, Kim TS, Han SY, et al. 2007. Suppressive effects of bisphenol a on the proliferation of neural progenitor cells. J Toxicol Environ Health A 70:1288-1295.
Kitajima TS, Sakuno T, Ishiguro K, Iemura S, Natsume T, Kawashima SA, et al. 2006. Shugoshin collaborates with protein phosphatase 2a to protect cohesin. Nature 441:46-52.
Kollareddy M, Dzubak P, Zheleva D, Hajduch M. 2008. Aurora kinases: Structure, functions and their association with cancer. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 152:27-33.
Laemmli UK. 1970. Cleavage of structural proteins during assembly of head of bacteriophage-t4. Nature 227:680-&.
LaPensee EW, Tuttle TR, Fox SR, Ben-Jonathan N. 2009. Bisphenol a at low nanomolar doses confers chemoresistance in estrogen receptor-alpha-positive and -negative breast cancer cells. Environ Health Perspect 117:175-180.
Lenie S, Cortvrindt R, Eichenlaub-Ritter U, Smitz J. 2008. Continuous exposure to bisphenol a during in vitro follicular development induces meiotic abnormalities. Mutat Res 651:71-81.
Li D, Zhou Z, Qing D, He Y, Wu T, Miao M, et al. 2010. Occupational exposure to bisphenol-a (bpa) and the risk of self-reported male sexual dysfunction. Hum Reprod 25:519-527.
Loh JK, Lieu AS, Chou CH, Lin FY, Wu CH, Howng SL, et al. 2010. Differential expression of centrosomal proteins at different stages of human glioma. BMC Cancer 10:268.
Naciff JM, Khambatta ZS, Reichling TD, Carr GJ, Tiesman JP, Singleton DW, et al. 2010
The genomic response of ishikawa cells to bisphenol a exposure is dose- and time-dependent. Toxicology 270:137-149.
Nakagomi M, Suzuki E, Usumi K, Saitoh Y, Yoshimura S, Nagao T, et al. 2001. Effects of endocrine disrupting chemicals on the microtubule network in chinese hamster v79 cells in culture and in sertoli cells in rats. Teratog Carcinog Mutagen 21:453-462.
Ochi T. 1999. Induction of multiple microtubule-organizing centers, multipolar spindles and multipolar division in cultured v79 cells exposed to diethylstilbestrol, estradiol-17beta and bisphenol a. Mutat Res 431:105-121.
Otto C, Rohde-Schulz B, Schwarz G, Fuchs I, Klewer M, Brittain D, et al. 2008. G protein-coupled receptor 30 localizes to the endoplasmic reticulum and is not activated by estradiol. Endocrinology 149:4846-4856.
Pacchierotti F, Ranaldi R, Eichenlaub-Ritter U, Attia S, Adler ID. 2008. Evaluation of aneugenic effects of bisphenol a in somatic and germ cells of the mouse. Mutat Res 651:64-70.
Parry EM, Parry JM, Corso C, Doherty A, Haddad F, Hermine TF, et al. 2002. Detection and characterization of mechanisms of action of aneugenic chemicals. Mutagenesis 17:509-521.

Pfeiffer E, Rosenberg B, Deuschel S, Metzler M. 1997. Interference with microtubules and induction of micronuclei in vitro by various bisphenols. Mutat Res 390:21-31.
Ptak A, Wrobel A, Gregoraszczuk EL. 2011. Effect of bisphenol-a on the expression of selected genes involved in cell cycle and apoptosis in the ovcar-3 cell line. Toxicol Lett 202:30-35.
Raynaud-Messina B, Merdes A. 2007. Gamma-tubulin complexes and microtubule organization. Curr Opin Cell Biol 19:24-30.
Ricupito A, Del Pozzo G, Diano N, Grano V, Portaccio M, Marino M, et al. 2009. Effect of bisphenol a with or without enzyme treatment on the proliferation and viability of mcf-7 cells. Environ Int 35:21-26.
Rivera T, Ghenoiu C, Rodriguez-Corsino M, Mochida S, Funabiki H, Losada A. 2012. Xenopus shugoshin 2 regulates the spindle assembly pathway mediated by the chromosomal passenger complex. Embo J 31:1467-1479.
Sekizawa J. 2008. Low-dose effects of bisphenol a: A serious threat to human health? Journal of Toxicological Sciences 33:389-403.
Shanle EK, Xu W. 2010. Endocrine disrupting chemicals targeting estrogen receptor signaling: Identification and mechanisms of action. Chem Res Toxicol 24:6-19.
Shi T, Dong F, Liou LS, Duan ZH, Novick AC, DiDonato JA. 2004. Differential protein profiling in renal-cell carcinoma. Mol Carcinog 40:47-61.
Stahlhut RW, Welshons WV, Swan SH. 2009. Bisphenol a data in nhanes suggest longer than expected half-life, substantial nonfood exposure, or both. Environ Health Perspect 117:784-789.
Takada Y, Kato C, Kondo S, Korenaga R, Ando J. 1997. Cloning of cdnas encoding g protein-coupled receptor expressed in human endothelial cells exposed to fluid shear stress. Biochem Biophys Res Commun 240:737-741.
Tanno Y, Kitajima TS, Honda T, Ando Y, Ishiguro K, Watanabe Y. 2010. Phosphorylation of mammalian sgo2 by aurora b recruits pp2a and mcak to centromeres. Genes Dev 24:2169-2179.
Thomas P, Dong J. 2006. Binding and activation of the seven-transmembrane estrogen receptor gpr30 by environmental estrogens: A potential novel mechanism of endocrine disruption. J Steroid Biochem Mol Biol 102:175-179.
Toth B, Saadat G, Geller A, Scholz C, Schulze S, Friese K, et al. 2008. Human umbilical vascular endothelial cells express estrogen receptor beta (er beta) and progesterone receptor a (pr-a), but not er alpha and pr-b. Histochemistry and Cell Biology 130:399-405.
Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. 2007. Human exposure to bisphenol a (bpa). Reproductive Toxicology 24:139-177.
Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM. 2009. Bisphenol-a and the great divide: A review of controversies in the field of endocrine disruption. Endocrine Reviews 30:75-95.
Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgartten FJ, Schoenfelder G. 2010. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol a. Environ Health Perspect 118:1055-1070.
Welshons WV, Nagel SC, vom Saal FSV. 2006. Large effects from small exposures. Iii. Endocrine mechanisms mediating effects of bisphenol a at levels of human exposure. Endocrinology 147:S56-S69.
Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenscheing C, et al. 2007. In vitro molecular mechanisms of bisphenol a action. Reproductive Toxicology 24:178-198.
Yuan J, Pu MT, Zhang ZG, Lou ZK. 2009. Histone h3-k56 acetylation is important for genomic stability in mammals. Cell Cycle 8:1747-1753.

3 The Environmental Pollutant Bisphenol A Interferes with Nucleolar Structure



Edna Ribeiro-Varandas, H Sofia Pereira, Sara Monteiro, Ricardo Boavida Ferreira, Elsa Neves, Luisa Brito, Wanda Viegas, Margarida Delgado. 2012. "The Environmental Pollutant Bisphenol A Interferes with Nucleolar Structure", 2012 International Conference on Biomedical Engineering and Biotechnology, icbeb, pp.1811-1814

3.1 Abstract

Bisphenol A (BPA) is an environmental pollutant to which humans are universally exposed, evident as low BPA concentrations present in biological samples in the majority of individuals in developed countries. Although BPA is widely detected in blood, the main source of human exposure is through ingestion, as BPA is broadly used in the plastic industry and therefore present in food containers. In this work, Umbilical Vascular Endothelial Cells (HUVEC) and Human Colon Adenocarcinona (HT29) cell lines were utilized to evaluate the effects of low BPA concentrations on nucleolar organization and function. Our results show that BPA levels commonly found in humans can affect several nucleolar features, including fibrillarin distribution and mean nucleoli number, nucleolin gene expression and nucleoli epigenetic marks.

3.2 Introduction

BPA is an extensively utilized industrial Endocrine Disrupting Chemical (EDC) with the ability to behave as biological signals and interfere with endogenous hormone functions, and is employed in the manufacture of an outstanding variety of consumer products (Wetherill et al. 2007). It has been shown that xenobiotics exposure can endorse changes in nucleolus organization and protein content in response to signalling pathways activated by the cellular stress response (Boulon et al. 2010). However, the effects of BPA on the nucleolus is still poorly understood, although anomalies associated to BPA exposure including nucleolar fragmentation have been reported in salmon cells (Honkanen et al. 2004). Transcription of rRNA genes occurs in the nucleoli dense fibrillar component that also comprises fibrilarin and nucleolin proteins, two of the most abundant non-ribosomal proteins of the nucleolus (Bartova et al. 2010; Mongelard and Bouvet 2007). In this work, we evaluated the effects of BPA on fibrillarin organization, 18 rRNA and nucleolin gene transcription as well as H3 epigenetic marks associated to gene regulation. Two BPA concentrations were assayed, 10 ng/ml, which is within the concentration range found in humans (Vandenberg et al. 2010) as well as 1 μg/ml.

Two distinct cell lines HUVEC and HT29 cell lines representative of vascular and digestive tract tissues which are in direct contact to BPA *in vivo* were utilized (Vandenberg et al. 2007).

3.3 Materials and methods

Cell cultures, reagents and treatments

HT29 human cell line was cultivated in 75cm^2 flasks with RPMI media containing GlutaMAX™ I, 25 mM HEPES (Gibco), supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. HUVEC cell line was cultivated in 75cm^2 flasks coated with 0.2% gelatin, grown in EGM-2 media supplemented with BulletKit (Lonza), 5% (v/v) fetal bovine serum and 0,2% (v/v) Bovine Brain Extract. Cell cultures were maintained in a humidified 5% (v/v) CO2 atmosphere at 37°C. For BPA treatments, BPA was freshly diluted in ethanol and added to the culture media to final concentrations of 10 ng/ml (44 nM) or 1 µg/ml (4.4 µM). BPA was added 24 h after cultivation initiation and cells were incubated in BPA supplemented medium for an additional 72 h. Cells were also grown in standard culture media (control) and standard culture media supplemented with ethanol corresponding to the final concentration of ethanol in BPA treated cultures (0.17 mM-vehicle) as controls.

Immunofluorescence

Cells were plated in Petri dishes containing glass coverslips coated with 0.2% (v/v) gelatin (Sigma). After treatments, cells were fixed, permeabilized, and incubated in 5% (w/v) BSA/PBS solution for 60 min followed by incubation with the primary antibodies diluted in 1% (w/v) BSA /PBS. After washing, conjugated anti-rabbit-FITC IgG (1:200, Abcam) or anti-mouse-Cy3 IgG (1:200, Sigma) secondary antibodies were added and incubated for 60 min at 37°C. Cells were then washed, DAPI stained, and coverslips mounted on glass slides with antifade AF1 (Citifluor). ImageJ software (http://rsbweb.nih.gov/ij/) was used for image analysis, and a minimum of 25 cells per growth condition were analyzed for at least two separate experiments.

Protein extraction and western blotting analysis

Cells at 80% confluence were collected by trypsinization, pellets were resuspended in 500 µl SDS buffer and sonicated on ice. Protein concentrations were determined by the Bradford method. Western blotting electrophoresis on polyacrylamide gel was performed as described in (Laemmli 1970) using 50 µg total protein samples transferred to PVDF membranes and stained by PonceauS reagent. Immunoblots were blocked, incubated with primary antibodies, and detected with SuperSignal West Femto Maximum sensitivity substrate kit solution. Immunoreactive protein bands are visualized with the BioRad Chemidoc XRS.

cDNA isolation and real-time quantitative PCR (qRT-PCR)

Cells were collected at 80% confluence and RNA isolated with the RNAqueous Kit (BioPortugal). After RNase free DNase digestion (RQ1 RNase free DNase) cDNA synthesis was performed with random primers using DYNAmo cDNA syntesis Kit. cDNA was utilized for qRT-PCR with the BIO-RAD SsoFast Eva Green Supermix. The genes analyzed and their respective primer sequences are shown in the table 1. Experiments were repeated at least twice with a minimum of three replicates per cell treatment/primer combination in each experiment. Threshold cycles (C_t) were equilibrated with GAPDH to calculate ΔC_t (ΔC_t = C_t gene – mean GAPDH C_t). Gene expression levels were analyzed by calculating $\Delta\Delta C_t$ ($\Delta\Delta C_t$ = ΔC_t treatment – mean ΔC_t vehicle), which in turn was used to determine mean fold change ($2^{-\Delta\Delta Ct}$) ± standard deviation.

Table 1. Primers used for qRT-PCR

Gene	Accession nº	Forward primer (5´→3´)	Reverse primer (5´→3´)
18S rDNA	NR_003286	CATTCGAACGTCTGCCCTAT	CCTCCAATGGATCCTCGTTA
GAPDH	NM_002046	GAGTCAACGGATTTGGTCGTA	GCAGAGATGATGACCCTTTTG
NCL	NM_005381	CCTTCTGAGGACATTCCAAGACA	ACGGTATTGCCCTTGAAATGTT

[a] GenBank accession numbers (National Center for Biotechnology).

Statistical analysis

Cells grown in standard medium (control) were compared to cells exposed to vehicle (ethanol) or BPA treatments and mean expression values compared with Student's t Test.

3.4 Results

3.4.1 Nucleolar organization is affected by BPA exposure.

Fibrillarin interphase distribution patterns were evaluated for each growth condition by immunofluorescence (Figure 1). No differences were observed between control and vehicle, where fibrillarin showed a typical heterogeneous nucleolar distribution at interphase (Figure 1A). However on BPA exposed cells, there was a significant reduction in signal dispersal observed as an overall reduction in the ratios of fibrillarin signal area/nuclear as calculated using ImageJ software (Figure 1B). Relative fibrilarin area was reduced in HUVEC from 0.102 ± 0.022 in control, to 0.078 ± 0.017 for 10 ng/ml and 0,073 ± 0,02 for 1 µg/ml BPA treated cells. In HT29, this reduction was from 0.129 ± 0.026 in control cells to 0.099 ± 0.023 for 10 ng/ml and 0.136 ± 0.023 for 1 µg/ml BPA (Figures 1A and 1B). HUVEC cells therefore presented a decrease of relative fibrillarin area at both BPA concentrations assayed, whereas HT29 cells showed a significant decrease of relative fibrillarin area exclusively in cells exposed to the lower BPA concentration (Figure 1B). We also observed an overall decrease in average number of nucleoli, evident as a significant reduction from 4,14 ± 1,08 nucleoli in control HUVEC cells to 3,4 ± 0,95 for 10 ng/ml and 3,3 ± 0,77 for 1 µg/ml BPA treated HUVEC, and from 2,05 ± 0,7 in controls to 1,42 ± 0,49 for 10 ng/ml and 1,33 ± 0,47 for 1 µg/ml BPA treated HT29 (Figure 1C). Interestingly, considering that both the overall fibrillarin area and number of nucleoli decreased in BPA treatments, no differences were found in total fibrillarin content (Figure 1D).

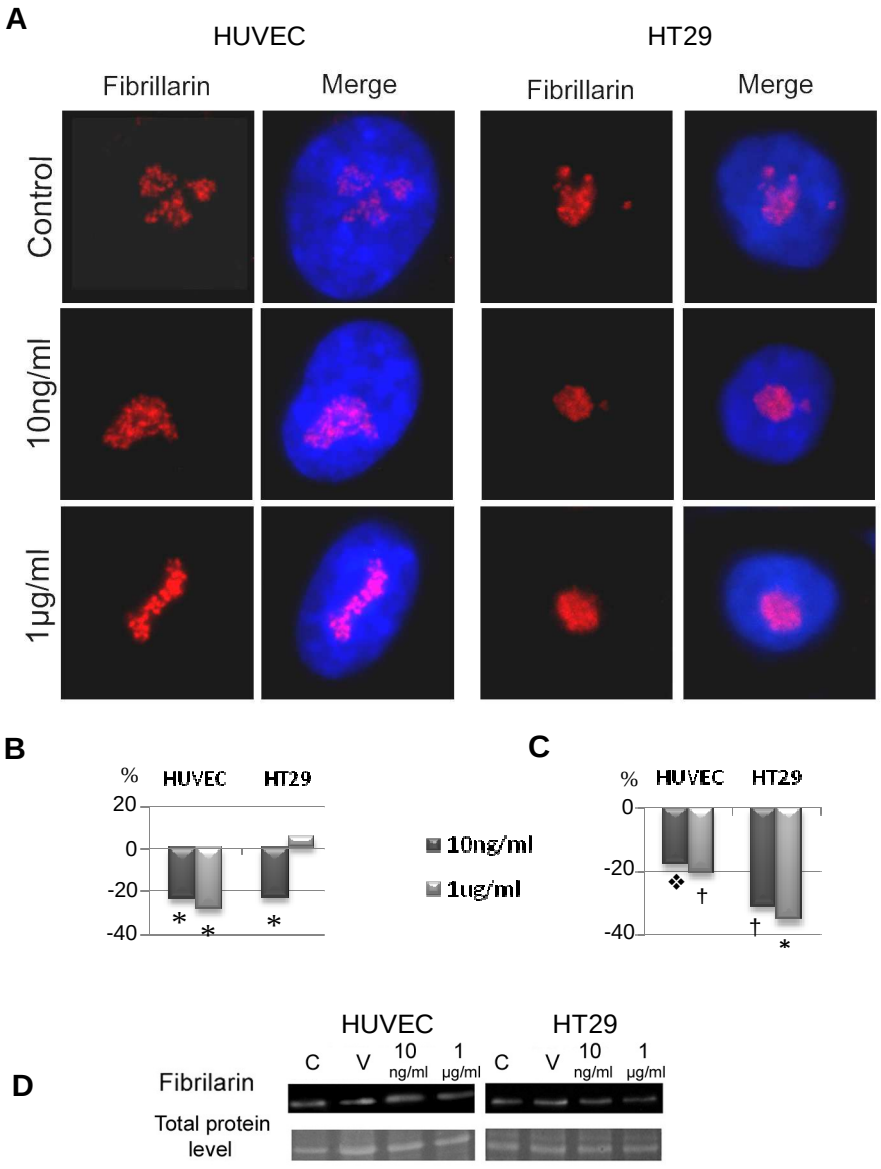


Figure 1 - Effects of BPA on fibrillarin in HUVEC and HT29 (A) Interphase nuclei after fibrillarin immunodetection (red) in control and after exposure to BPA at 1 µg/ml or at 10 ng/ml. Merged images with DAPI staining are shown on the right, bar = 5µm. (B) Variation in mean number of nucleoli, and (C) variation in relative fibrillarin area. In (B) and (C) results are presented as % of variation in relation to control and Student's t Tests significance levels are shown ❖p < 0.05; †p < 0.01 and *p < 0.001 (D) Fibrillarin Western blotting revealing an identical band of approximately 40 KDa evident for all treatments. Bottom panel shows total protein used as loading controls.

3.4.2 BPA effects on nucleolin transcription are cell specific and independent of rRNA levels.

Nucleolin gene (*NCL*) and 18S rDNA transcription levels were analyzed by qRT-PCR in HUVEC and HT29 cells (Figure 2). No significant differences were observed for 18S rRNA levels independently of cell line or BPA concentration. Conversely, a cell specific effect was detected for *NCL* expression levels, where a significant increase of *NCL* transcripts at both BPA concentrations was observed exclusively in HUVEC cells. These results indicate that BPA effects on *NCL* levels depend on cell type and are independent of rRNA levels.

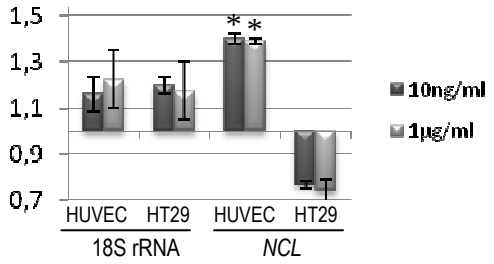


Figure 2 - Analysis of 18S rDNA and NCL transcript levels by quantitative real-time PCR on HUVEC and HT29 cells exposed to BPA at 1 µg/ml or 10 ng/ml. PCR threshold cycles (Ct) were equilibrated with GAPDH and results are shown as mean fold change ($2^{-\Delta\Delta Ct}$) ± standard deviation in relation to vehicle, *p < 0.001.

3.4.3 Bisphenol A alters H3 epigenetic marks at the nucleolar region.

Since nucleolar organization is closely associated to several epigenetic factors implicated in gene regulation (Bartova et al. 2010) we evaluated BPA effects on the distribution histone H3 modified in two distinct lysine residues, namely bi-methylated lysine 9 (H3K9me2) and tri-methylated lysine 4 (H3K4me3) on HUVEC cells (Figure 3A). Although overall nuclear distribution of these epigenetic marks were not affected by BPA exposure, decreased levels of H3K9me2 accompanied by increased levels of H3K4me3 were observed in the nucleolus of BPA treated cells (Figure 3A, compare top and bottom panels). These results were more marked at 1 µg/ml of BPA exposure, suggesting a dose dependent effect. Immunoblotting on total protein extracts revealed that H3K9me2 and H3K4me3 variation at the nucleoli area is not due to total protein levels (Figure 3B), evident as identical low intensity immunoreactive protein bands with the expected size (~17 kDa) for both H3 modifications.

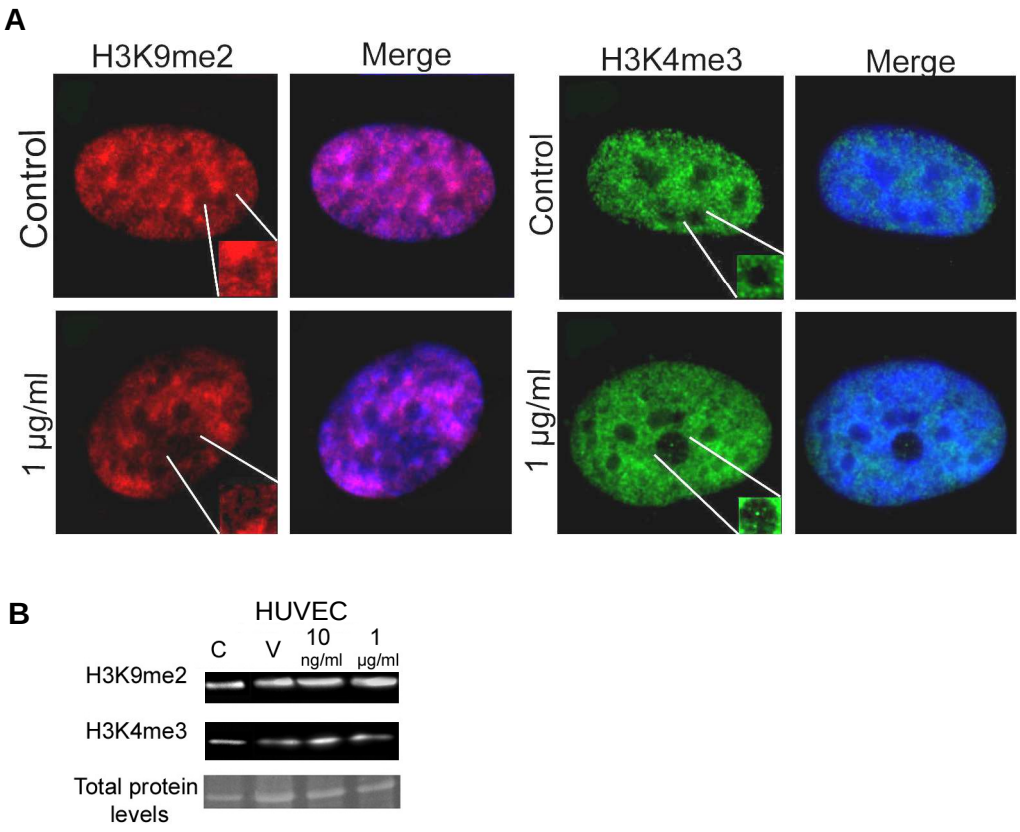


Figure 3 - BPA effects on modified histones, H3K9me2 and H3K4me3. (A) HUVEC nuclei showing distribution of epigenetic marks H3K9me2 (red) and H3K4me3 (green) in controls and cells treated with 1 µg/ml of BPA. Enlargements of the nucleolar region are shown in insets. DNA was stained with DAPI (blue) and Merged images are shown on the right, bars = 5 µm. (B) Western blotting on total protein extracts result in H3K9me2 and H3K4me3 specific bands corresponding to approximately 17 KDa. Total protein levels used as loading control are shown.

3.5 Discussion

Toxicological assessment of epigenetic changes is of particular importance, as these alternations can have permanent effects on gene expression patterns and be passed on transgenerationaly (Crews and McLachlan 2006). Special attention has been given to toxic effects of EDCs, such as the estrogenic compound BPA, to which human exposure is considered generalized in developed countries (Vandenberg et al. 2009). BPA can simultaneously and differentially trigger specific signaling pathways in different cell types, responsible for the nature and magnitude of biological responses (Dong et al.

2010). The importance of assessing toxicological effects on nucleolar organization and epigenetic marks has become exceedingly obvious over the last years (Lebaron et al. 2010; Szyf 2007). The nucleolus is one of the main targets of signalling pathways activated by the cellular stress response (Magakian et al. 2009), however the existent information regarding BPA effects on nucleolar organization and function is still extremely scarce. In this work, we show that BPA has a number of effects on the nucleolus, a nuclear compartment involved in numerous essential cellular processes. Immunocytofluorescence with the nucleolar marker fibrillarin shows that BPA exposure results in a reduction of active nucleolar regions in both studied cell lines. This indicates that BPA is capable of nucleolar disruption and interfering with mechanisms of nucleolar architecture and organization. It should be emphasized that these effects were also observed in cells exposed to the lower BPA concentration used in this work (10 ng/ml), which it is within the range of BPA levels found in humans as a result of common environmental exposure (Vandenberg et al. 2010). We observe a decrease in the relative area of fibrillarin in HUVEC cells for both BPA treatments assayed, while this effect was only observed in HT29 cells exposed to the lowest BPA concentration. This not only reveals that BPA has differential effects on distinct cells, but more importantly, demonstrates that extremely low BPA dosages can trigger alterations that are not detected in higher dosages. In addition, BPA induces up-regulation of nucleolin mRNA levels exclusively on HUVEC cells independently of rDNA transcription. Like fibrillarin, nucleolin is an integral protein of the dense fibrillar nucleolar component involved in several major cellular functions (Bartova et al. 2010). Finally, BPA induces alterations on epigenetic marks H3K9me2 and H3K4me3, associated respectively to transcriptional silencing and transcriptional competence (Bartova et al. 2010). The observed decreased nucleolar H3K9me2 and increased nucleolar H3K4me3 levels suggest that BPA induces a more a permissive transcriptional environment in the nucleolus. In accordance, decreased H3K9me2 nucleolar levels have been observed in cells exposed to the DNA hypomethylation agent 5-azacytitine (Bartova et al. 2010) and BPA has been shown to induce DNA hypomethylation in animals (Bromer et al. 2010; Dolinoy et al. 2007). This suggests that the observed changes in

nucleolar epigenetic marks might be mediated by a reduction in the methylation status of nucleolar associated chromatin. Taken together, our results showing numerous effects of BPA on nucleolar structure support the increasing concern regarding BPA risk assessment (reviewed in Vandenberg et al., 2009).

3.6 References

Bartova E, Horakova AH, Uhlirova R, Raska I, Galiova G, Orlova D, et al. 2010. Structure and epigenetics of nucleoli in comparison with non-nucleolar compartments. J Histochem Cytochem 58:391-403.

Boulon S, Westman BJ, Hutten S, Boisvert FM, Lamond AI. 2010. The nucleolus under stress. Mol Cell 40:216-227.

Bromer JG, Zhou YP, Taylor MB, Doherty L, Taylor HS. 2010. Bisphenol-a exposure in utero leads to epigenetic alterations in the developmental programming of uterine estrogen response. Faseb Journal 24:2273-2280.

Crews D, McLachlan JA. 2006. Epigenetics, evolution, endocrine disruption, health, and disease. Endocrinology 147:S4-S10.

Dolinoy DC, Huang D, Jirtle RL. 2007. Maternal nutrient supplementation counteracts bisphenol a-induced DNA hypomethylation in early development. Proceedings of the National Academy of Sciences of the United States of America 104:13056-13061.

Dong S, Terasaka S, Kiyama R. 2010. Bisphenol a induces a rapid activation of erk1/2 through gpr30 in human breast cancer cells. Environ Pollut 159:212-218.

Honkanen JO, Holopainen IJ, Kukkonen JV. 2004. Bisphenol a induces yolk-sac oedema and other adverse effects in landlocked salmon (salmo salar m. Sebago) yolk-sac fry. Chemosphere 55:187-196.

Laemmli UK. 1970. Cleavage of structural proteins during assembly of head of bacteriophage-t4. Nature 227:680-&.

Lebaron MJ, Rasoulpour RJ, Klapacz J, Ellis-Hutchings RG, Hollnagel HM, Gollapudi BB. 2010. Epigenetics and chemical safety assessment. Mutat Res.

Magakian YA, Karalyan ZA, Karalova EM, Abroyan LO, Akopyan LA, Gasparyan MH, et al. 2009. Comparative multiparametric analysis of hela and rd cell culture reactions to solcoseryl. Bulletin of Experimental Biology and Medicine 148:615-618.

Mongelard F, Bouvet P. 2007. Nucleolin: A multifaceted protein. Trends Cell Biol 17:80-86.

Stahlhut RW, Welshons WV, Swan SH. 2009. Bisphenol a data in nhanes suggest longer than expected half-life, substantial nonfood exposure, or both. Environ Health Perspect 117:784-789.

Szyf M. 2007. The dynamic epigenome and its implications in toxicology. Toxicological Sciences 100:7-23.

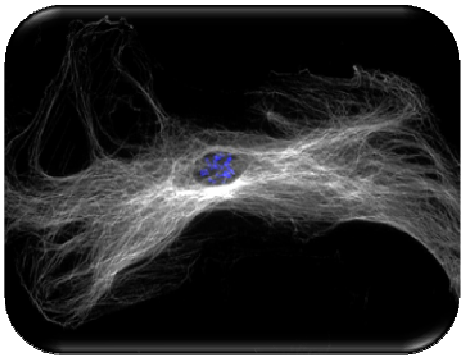
Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. 2007. Human exposure to bisphenol a (bpa). Reproductive Toxicology 24:139-177.

Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM. 2009. Bisphenol-a and the great divide: A review of controversies in the field of endocrine disruption. Endocrine Reviews 30:75-95.

Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgartten FJ, Schoenfelder G. 2010. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol a. Environ Health Perspect 118:1055-1070.

Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenscheing C, et al. 2007. In vitro molecular mechanisms of bisphenol a action. Reproductive Toxicology 24:178-198.

4 Bisphenol A disrupts transcription and decreases viability in aging vascular endothelial cells



Edna Ribeiro-Varandas, H Sofia Pereira, Sara Monteiro, Ricardo Boavida Ferreira, Elsa Neves, Luisa Brito, Wanda Viegas, Margarida Delgado. Bisphenol A disrupts transcription and decreases viability in aging vascular endothelial cells. Accepted for publication in International Journal of Molecular Sciences, manuscript ID: ijms-40153, 2014.

4.1 Abstract

Bisphenol A (BPA) is a widely utilized endocrine disruptor capable of mimicking endogenous hormones, employed in the manufacture of numerous consumer products, thereby interfering with physiological cellular functions. Recent research has shown that BPA alters epigenetic cellular mechanisms in mammals and may be correlated to enhanced cellular senescence. Here, the effects of BPA at 10 ng/mL and 1 µg/mL, concentrations found in human samples, were analyzed on HT29 human colon adenocarcinona cell line and Human Umbilical Vein Endothelial Cells (HUVEC). Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) transcriptional analysis of the Long Interspersed Element-1 (LINE-1) retroelement showed that BPA induces global transcription deregulation in both cell lines, although with more pronounced effects in HUVEC cells. Whereas there was an increase in global transcription in HT29 exclusively after 24 h of exposure, this chemical had prolonged effects on HUVEC. Immunoblotting revealed that this was not accompanied by alterations in the overall content of H3K9me2 and H3K4me3 epigenetic marks. Importantly, cell viability assays and transcriptional analysis indicated that prolonged BPA exposure affects aging processes in senescent HUVEC. To our knowledge this is the first report that BPA interferes with senescence in primary vascular endothelial cells, therefore, suggesting its association to the etiology of age-related human pathologies, such as atherosclerosis.

4.2 Introduction

Bisphenol A (BPA) is an industrial chemical, employed in the manufacture of many consumer products such as polycarbonate plastics and epoxy resins. The main source of human exposure to BPA is due to leaching from containers resulting in its ingestion (Ballesteros-Gomez et al. 2009; Mezcua et al. 2012). Biomonitoring studies have showed that environmental exposure results in detectable internal BPA levels in the majority of individuals analyzed, (reviewed in Vandenberg et al. 2010) and particularly high levels associated with occupational exposure (He et al. 2009; Li et al. 2010). BPA is an environmental

xenoestrogen capable of triggering distinct estrogen-signalling pathways with potential consequences for human health, (reviewed in Rubin 2011). Several studies show that BPA at very low concentrations alters proliferation kinetics and expression of cell cycle related genes, (Ptak et al. 2011; Ribeiro-Varandas et al. 2013). Moreover, BPA exposure can affect DNA methylation and consequently gene expression, (reviewed in Singh and Li 2012). This was first observed in mice where BPA induced hypomethylation of the intracisternal A particle (IAP) retrotransposon results in altered expression of associated genes (Dolinoy et al. 2007). Furthermore genome wide analysis of DNA methylation in the developing mouse forebrain revealed that 64% of the loci analyzed had developmental stage-dependent hyper or hypo methylation alterations associated with BPA exposure (Yaoi et al. 2008). Altered transcription patterns associated with modifications in DNA methylation have also been reported for several genes in human cells exposed to BPA (Fernandez et al. 2012). Although there is substantial evidence that BPA affects DNA methylation, the effects of this chemical on histone modifications remain largely unknown, (reviewed in Singh and Li 2012). One report revealed that increased expression of the histone methyltransferase enhancer of Zeste Homolog 2 (*EZH2*) is induced by BPA with consequent increase in the overall level of histone H3 trimethylation at lysine 27 (H3K27me3), both in human breast cancer cells and in mouse mammary glands (Doherty et al. 2010). Recently, we have also shown that BPA alters intranucleolar distribution of two distinct H3 post-translation modifications (H3K4me3 and H3K9me2) in primary human endothelial cells (HUVEC), although without alteration in the global level of these two epigenetic marks (Ribeiro-Varandas E. 2012).

Numerous studies have established a close relationship between epigenetics and aging. Epigenetic modifications have been highly correlated with age-related pathologies such as cancer, neurodegenerative and cardiovascular disorders, as wells as physiological processes of aging itself, (reviewed in Boyd-Kirkup et al. 2013; D'Aquila et al. 2013). Cellular aging is characterized by continuous physiological alterations and loss of replicative capacity. In primary cell culture, after a number of population doublings in cell cultures, actively dividing cell numbers decrease as cells enter replicative senescence.

Senescence can also be induced by exposure to subcytotoxic doses of stressful agents such as oxidants or ethanol (Dumont et al. 2002; Toussaint et al. 2000). Accordingly, it has been recently shown that BPA may have an important role in enhanced cellular senescence in normal human mammary epithelial cells (HMEC), attributable to deregulation of cell cycle regulatory genes (Qin et al. 2012). A relevant fact is that high urinary BPA concentrations have been correlated with pathogenesis of age-related diseases such as coronary and carotid atherosclerosis (Lind and Lind 2011; Melzer et al. 2010; Melzer et al. 2012), a form of accelerated arterial senescence (Andreassi 2008). However, despite the extensive research on BPA effects in human cancer cells, effects of continuous contact on vascular primary cells are mostly unknown.

In this work, we utilized HUVEC and HT29 human colon adenocarcinona cell line to compare BPA effects on primary cells of vascular tissue and cancer cells from digestive tract tissue. The Long Interspersed Element-1 (LINE-1 or L1) is a highly abundant retroelement distributed throughout the human genome. To access BPA effects on HT29 and HUVEC cells global transcription, we evaluated the expression of two distinct regions of LINE-1 (Aporntewan et al. 2011). In addition, BPA effects on cellular viability and expression of senescence associated genes were analyzed on aging HUVEC cells.

4.3 Materials and methods

Cell cultures and reagents

The effects of BPA were analyzed on HT29 and HUVEC representative of digestive and vascular tract tissues, respectively, which are exposed to BPA *in vivo*. HT29 human colon adenocarcinoma cell line was purchased from the European Collection of Cell Cultures (ECACC, London, UK) and was cultivated in 75 cm^2 flasks with RPMI media containing GlutaMAX™ (#61870010, Invitrogen, Carlsbad, CA, USA), supplemented with 10% (*w*/*v*) fetal bovine serum (#16000-044, Invitrogen), 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine. Primary vascular endothelial (HUVEC) cells were kindly provided by Neo-vascularization Lab (IMM, Lisbon, Portugal), and cultivated in 75 cm^2 flasks coated with 0.2% (*w*/*v*) gelatin. HUVEC were grown in EGM-2 media

supplemented with the respective specific BulletKit (#CC-3156 and #CC-4176, LONZA, Basel, Switzerland) and with 5% (*v*/*v*) fetal bovine serum and 0.2% (*w*/*v*) Bovine Brain Extract (#CC-4098, LONZA). All cell cultures were maintained in a humidified 5% (*v*/*v*) CO_2 in air atmosphere at 37 °C. For treatments and experiments, HUVEC were used between passages 3–6 (young HUVEC) and passages 11–19 (aging HUVEC). For aging cell passages were performed every 3–4 days independently of the confluence status and seeded at an identical density (3.2×10^3 cel/cm^2) for all conditions assayed.

BPA treatments and controls

After subculture procedure cells were stabilized for 24 h in standard growth medium before all experiments. BPA was freshly diluted in ethanol and added to the culture media to final concentrations of 10 ng/mL (44 nM) or 1 µg/mL (4.4 µM). As negative controls cells were grown in either standard culture media or in media supplemented with ethanol (vehicle) at a final concentration of 0.17 mM, corresponding to the vehicle added for both BPA concentrations assayed. For short exposure treatments, HT29, young (passages 4 and 6) and aging (passages 12) HUVEC were incubated in medium with BPA or control media for 24 or 72 h. For long and continuous BPA exposure, HUVEC were maintained in medium with or without BPA from passage 12 to passage 19. At each passage, cells were collected by trypsinization and cellular viability evaluated with trypan blue.

cDNA isolation and real-time quantitative PCR

Transcriptional analysis was performed by qRT-PCR for two distinct regions of the LINE1 retrolement (L1-3' and L1-5') and for osteonectin (*SPARC*), fibronectin (*FN1*), *p21*, *FOS*, *bcl-xL*, *NCL* and *18S rRNA* genes as well as control genes encoding *GAPDH* and *β-actin*, using the specific primers listed in Table 1.Total RNA was extracted from trypsinized cells with the RNAqueous Kit (#AM1912, Invitrogen) following manufacturers' instructions. After verifying concentration and integrity, 3 µg of total RNA was utilized for RNase free DNase digestion (RQ1 RNase free DNase, #M6101, Promega, Madison, WI, USA) and first strand cDNA synthesis was completed with random primers

(DYNAmo cDNA syntesis Kit, #F-470L, Thermo Scientific, Waltham, MA, USA). The resulting cDNA was utilized for qRT-PCR with SsoFast Eva Green Supermix (#172-5201, BioRad, Hercules, CA, USA) utilizing the following conditions; 95 °C–3 min, 35 cycles (95 °C–30 s, 55 °C–30 s), 72 °C–5 min 40 °C. To ensure that genomic DNA was completely absent prior to cDNA synthesis, PCRs were performed with 18S primers and 250 ng of DNase digested RNA. Control PCRs were also performed for both primer combinations without template. Transcriptional analysis experiments were repeated at least three times and in each experiment at least three technical replicates per cell treatment/primer combination were performed. All comparisons of expression levels were performed on identical cDNA dilutions. After denaturation curves were observed to ensure correct amplification products, threshold cycles (*C*t) were equilibrated with mean GAPDH and β-actin to calculate Δ*C*t (Δ*C*t = *C*t of interest – mean (GAPDH-β-actin) *C*t) since no significant differences were detected between the two reference genes. Gene expression levels were analyzed by calculating ΔΔ*C*t (ΔΔ*C*t = Δ*C*t a – mean Δ*C*t b, where medium a and b are being compared). Results are presented as log2 of the mean fold change ($2^{-\Delta\Delta Ct}$) ± standard deviation. Student's *t* test was used for statistical analysis.

Table 1 Primers used for qRT-PCR.

Gene	Accession nº	Forward primer (5´→3´)	Reverse primer (5´→3´)
L1-5´	M80340.1	GGCCAGTGTGTGTGCGCACCG	CCAGGTGTGGGATATAGTCTCGTGG
L1- 3´	M80340.1	CAGGAAGGGGAATATCACACTC	TGCGCTGCACCCACTAACTC
SPARC	NM_003118	CTGTGGGAGCTAATCCTG	GGGTGCTGGTCCAGCTGG
FN1	NM_002026	TGTGGTTGCCTTGCACGAT	GCTTGTGGGTGTGACCTGAGT
p21	NM_000389	CTGGAGACTCTCAGGGTCGAA	CCAGGACTGCAGGCTTCCT
FOS	NM_005252	AGGAGAATCCGAAGGGAAAG	CAAGGGAAGCCACAGACATC
bcl-xL	NM_001191	TTACCTGAATGACCACCTA	ATTTCCGACTGAAGAGTGA
NCL	NM_005381	CCTTCTGAGGACATTCCAAGACA	ACGGTATTGCCCTTGAAATGTT
18SrRNA	NR_003286	CATTCGAACGTCTGCCCTAT	CCTCCAATGGATCCTCGTTA
GAPDH	NM_002046	GAGTCAACGGATTTGGTCGTA	GCAGAGATGATGACCCTTTTG
β-actin	NM_001101	GGTCATCTTCTCGCGGTTGGCCTTGGGGT	CCCCAGGCACCAGGGCGTGAT

[a] GenBank accession numbers (National Center for Biotechnology).

Protein extraction and western blotting analysis

For total protein lysate, cell pellets of trypsinized cultures were resuspended in 500 µL SDS buffer (0.125 M Tris–HCl, 10% (*v*/*v*) 2-mercaptoethanol, 2% (*w*/*v*)

SDS and 10% (*w*/*v*) sucrose) (Dong et al. 2011) and sonicated on ice 2 x 15 s. After centrifugation (14000 *g*), the supernatant was transferred to a fresh centrifuge tube and stored at −20 °C. Protein concentration was determined by the Bradford method (Protein assay, #500-0006, BioRad) and electrophoresis on polyacrylamide gels was performed using 50 µg of protein samples, transferred onto polyvinylidene difluoride membranes (PVDF) membranes, and stained by PonceauS reagent. The immunoblots were blocked with 3% (*w*/*v*) dry milk in PBST (0.05% (*v*/*v*) Tween 20, 137 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl) and incubated with primary antibodies: anti-trimethyl-histone H3 (Lys 4) (#ab8580, dilution 1:500; Abcam, London, UK) or anti-dimethyl-histone H3 (Lys 9) (#ab1220, dilution 1:2000; Abcam), and anti-α-tubulin (#T9026, dilution 1:2000; Sigma-Aldrich, St. Louis, MO, US). Secondary peroxidase-conjugated anti-rabbit and anti-mouse antibodies (#32460 and #32430, respectively; Pierce Biotechnology, Rockford, IL, USA) were used (dilutions 1:1250). Detection was performed with SuperSignal West Femto Maximum sensitivity substrate kit (#34096, Thermo Scientific) and BioRad Chemidoc XRS. Two independent protein extractions for each treatment were performed and Western blots repeated for reproducibility.

Cell viability assay

Cell viability assessment was performed on 96-well dishes using CellTiter-Blue Viability Assay (#G8081, Promega) according to manufacturer's instructions. After 4 h incubation fluorescence emission at 590 nm was measured using BioTek microplate reader Synergy HT. Cultivation and viability assay was performed simultaneously for all growth conditions. Statistic analysis was performed comparing treated cells with cells grown in standard medium. At least three independent experiments were performed with three replicates each per treatment. Student's *t* test was used for statistical analysis.

Immunofluorescence

Cells were grown over glass coverslips coated with 0.2% (*w*/*v*) gelatin. After treatments, cells were fixed in 4% (*v*/*v*) paraformaldehyde for 10 min at room temperature and permeabilized with 0.25% (*v*/*v*) Triton X-100 for 15 min. Fixed

cells were then incubated in 5% (*w*/*v*) BSA/PBS solution for 60 min and then incubated overnight at 4 °C with α-tubulin primary antibody (# ab7291, Abcam) diluted 1:200 in 1% (*w*/*v*) BSA/PBS. Detection was performed with conjugated anti-mouse-Cy3 IgG (#C2181, Sigma-Aldrich) secondary antibody diluted 1:200 in 1% (*w*/*v*) BSA/PBS for 60 min at 37 °C. Cells were then DAPI stained, and coverslips mounted on glass slides with antifade AF1 (Citifluor, London, UK). Immunofluorescence images were recorded with epifluorescence microscope Axioskop2 (Carl Zeiss, Oberkochen, Germany) equipped with a AxioCam MRc5 (Carl Zeiss) digital camera and superimposed with Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA) software.

4.4 Results and Discussion

4.4.1 Bisphenol A (BPA) Alters Global Transcription in HT29 Human Colon Adenocarcinona Cell Line and Human Umbilical Vein Endothelial Cells (HUVEC).

Considering that the main route of BPA exposure in humans occurs via ingestion followed by entrance into blood circulation, HT29 cell line and HUVEC were utilized as representatives of digestive tract and vascular tissues, respectively. BPA is extensively metabolized leading to biological inactivation, however, low levels of free BPA, the bioactive form, are found in human tissues and fluids (Vandenberg et al. 2010). Two distinct physiological relevant concentrations of free BPA were assayed, 10 ng/mL within the range found in human samples due to environmental exposure (Melzer et al. 2010; Vandenberg et al. 2010) and 1 μg/ml associated to occupational exposure (He et al. 2009; Li et al. 2010). BPA is known to affect the transcriptional expression of several genes involved in major cellular processes in different cell lines (Boehme et al. 2009; Bredhult et al. 2009; Buterin et al. 2006; Naciff et al. 2010). In order to assess the BPA effects on general transcription, the relative transcription of two sequences from the 5' and 3' regions of LINE-1 was evaluated. LINE-1 is a ~6 kb non-site-specific autonomous, non-Long Terminal Repeat retrotransposon that comprises approximately 17% of the human genome (Ostertag and Kazazian 2001) with more than 2500 L1 copies located in over 1400 genes (Aporntewan et al. 2011).

Quantitative RealTime-PCR (qRT-PCR) was performed with two sets of L1 specific primers (L1-5` and L1-3`) previously utilized for the evaluation of epigenetic gene regulatory mechanisms in cancer cells (Aporntewan et al. 2011). Transcriptional levels were compared between BPA and vehicle (mean log2 fold change ± standard deviation) in young HUVEC cells (passage 4, p4) as well as in the immortal cell line HT29 after 24 and 72 h treatments (Figure 1A, B). L1 transcription levels were significantly altered in HUVEC cells for both exposure times, however the early response (24 h) showed to be dose dependent with both sequences being up-regulated for the higher BPA concentration (1 µg/mL) (0.295 ± 0.165 for L1-5' and 0.55 ± 0.159 for L1-3'). Interestingly, after 72 h exposure, there was a significant decrease in the expression levels of L1-5' for 10 ng/mL BPA (−0.528 ± 0.279) and in both sequences for BPA at 1 µg/mL (−0.294 ± 0.14 for L1-5' and −1.176 ± 0.77 for L1-3') (Figure 1A). These results reveal that BPA affects 5' and 3' L1 sequences differentially in a dose dependent manner, particularly for the longer exposure time (72 h). Contrary to HUVEC, in HT29 cells L1 transcription was significantly increased exclusively after 24 h BPA exposure, at the lower BPA concentration assayed (0.315 ± 0.144 in L1-5' and 0.702 ± 0.479 in L1-3' for 10 ng/mL BPA). There were no effects in L1 transcription on HT29 cells exposed to BPA for 72 h (Figure 1B), suggesting that the HT29 response to BPA is lost with prolonged exposure.

Furthermore, there is evidence that BPA exposure affects DNA methylation of various genes (Fernandez et al. 2012) as well as retroelement sequences (Dolinoy et al. 2007). However information regarding BPA effects on histone modifications is almost inexistent (Singh and Li 2012). Here, we analyzed BPA effects on global content of two H3 histone modifications (H3K4me3 and H3K9me2) in HT29 cells. H3K4me3 and H3K9me2 are generally associated to gene activation and silencing, respectively (Hon et al. 2009). Immunoblotting of total protein extracts utilizing antibodies specific for each H3 modification revealed immunoreactive protein bands with the expected size of approximately 17 kDa (Figure 1C). No differences in the global content of modified histones were detected between BPA treatments and controls, as we have previously shown for HUVEC cells (Ribeiro-Varandas E. 2012). However, BPA induced

modifications of these epigenetic marks at particular genes cannot be excluded. Previous results have shown that BPA increases expression of histone methyl transferase EZH2 resulting in increased trimethylation of H3K27 (H3K27me3), a modification associated with transcriptional repression (Doherty et al. 2010). Considering that H3K4 and H3K9 methylation is independent of EZH2 (Varier and Timmers 2011), our results suggest that BPA differentially affects distinct histone methylation pathways.

L1 transcription analysis revealed that young primary HUVEC are more sensitive to BPA than HT29 cancer cells, in accordance with our previous results regarding BPA micronuclei induction and transcription alteration of chromosome segregation related genes (Ribeiro-Varandas et al. 2013). The less pronounced response to BPA in HT29 is not related with lack of expression of estrogen receptors, since both HUVEC and HT29 cells express classical estrogen receptor beta and HT29 expresses also the G protein-coupled estrogen receptor 1 (GPER) (Ribeiro-Varandas et al. 2013). It is important to notice that BPA affinity is considerably higher for GPER than for the classical ERs and recent studies have implicated this signaling pathway in BPA cellular response (Dong et al. 2011; Pupo et al. 2012). The lower responsiveness of HT29 may therefore be related with intrinsic features of cancer cells that render them more insensitive to external signals (Hanahan and Weinberg 2011). Given the higher BPA sensitivity of HUVEC and the fact that these are primary cells that undergo senescence in culture, BPA effects on L1 transcription in HUVEC at passage 12 (p12), *i.e.*, senescent cells were also analyzed(Khaidakov et al. 2011). Opposite BPA effects were detected for both L1 sequences with a decrease in L1-5' (−0.377 ± 0.221) and an increase in L1-3' transcriptional levels (0.722 ± 0.423) on aging HUVEC (Figure 1D), suggesting that this chemical may induce epigenetic modifications unevenly throughout the genome. Furthermore, these results show that the effects of BPA on L1 transcription differ depending on culture time, with aging cells having a pronounced response.

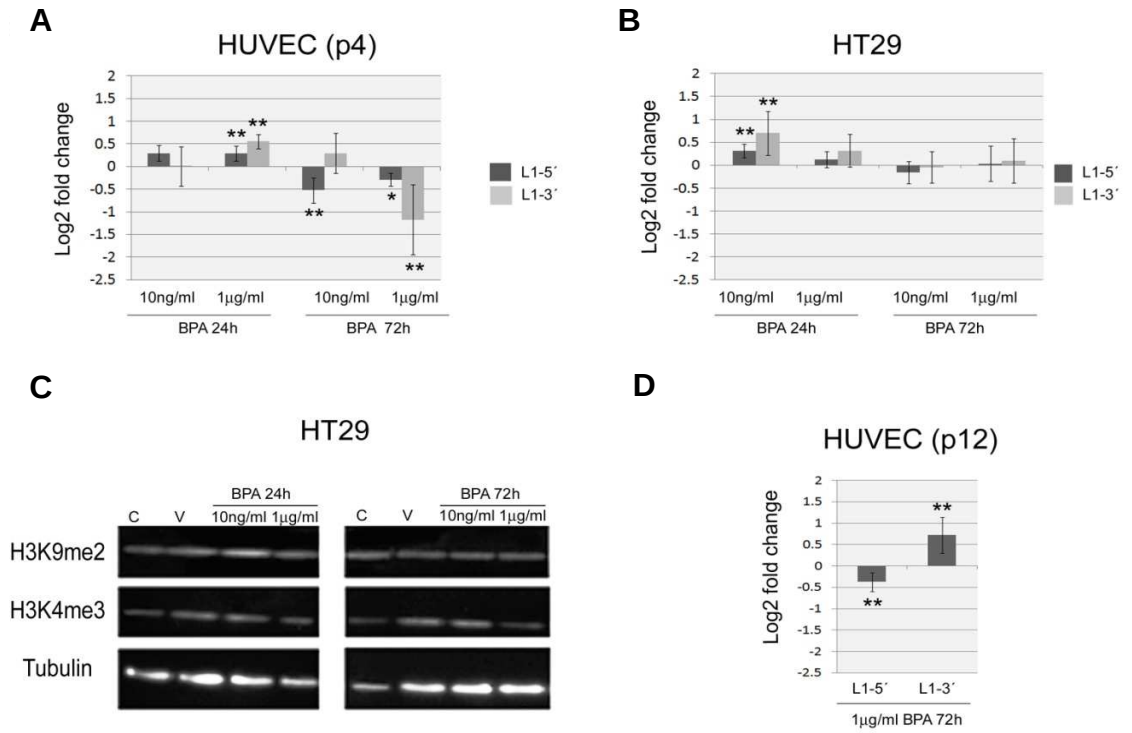


Figure 1 - Analysis of BPA effects on L1 transcription levels and H3K9me2 and H3K4me3 epigenetic marks. (**A, B**) L1-5' and L1-3' transcriptional analysis on young (passage 4, p4) Human Umbilical Vein Endothelial Cells (HUVEC) (**A**) and human colon adenocarcinona cell line HT29 (**B**) after 24 and 72 h exposure to 10 ng/mL or 1 µg/mL BPA; (**C**) Western blotting detection of H3 dimethylation at lysine 9 (H3K9me2) and H3 trimethylation at lysine 4 (H3K4me3) on HT29 cells after 24 and 72 h exposure to 10 ng/mL or 1 µg/mL BPA showing a single 17 kDa band identical to that observed for cells grown in control medium or in medium supplemented with vehicle, α-tubulin was used as loading control; (**D**) L1-5' and L1-3' transcription levels on aging HUVEC at passage 12 (p12) after 72 h exposure to 1 µg/mL BPA. Results in (**A**), (**B**), and (**D**) are shown as the mean log2 fold change ($2^{-\Delta\Delta Ct}$) ± standard deviation in relation to equivalent cells exposed to vehicle alone. Values significantly different from vehicle are indicated Student's t test **p < 0.01 and *p < 0.05.

4.4.2 Continuous BPA exposure reduces viability in aging HUVEC.

Despite the fact that human exposure to BPA is continuous throughout life, up to now a single study on mammary epithelial cells evaluated BPA effects in cellular senescence (Qin et al. 2012). Here, we analyzed BPA effects in cellular viability of aging HUVEC. At passage 12, HUVEC were exposed to BPA for 72 h or subjected to continuous BPA exposure for seven additional passages until passage 19 (p19). For comparison purposes, additional passages were performed regardless of the confluence level and cells were seeded at the same density for all passages/treatments. Equivalent cells were maintained for

the same periods in medium supplemented with vehicle. The results are presented in relation to cells maintained under standard conditions (Figure 2A). There were no considerable effects in cellular viability for vehicle (ethanol) in relation to control for any of the conditions assayed. More importantly, no significant variation in viability was detected after 72 h of BPA exposure at passage 12. Similarly at passage 15 (p15), corresponding to 384 h of continuous BPA exposure, no significant variation was detected between controls and treatments. Interestingly, at this passage a higher level of variation was detected independently of the growth medium, suggesting a transition phase in the senescence process. Conversely, both BPA concentrations tested induced a significant decrease in cellular viability for the longer periods, 528 h - passage 17 (p17) (−23.58% ± 4.80% and −26.60% ± 2.71%, for BPA 10 ng/mL and 1 µg/mL, respectively) and 693 h - passage 19 (−37.12% ± 1.83% and −26.26% ± 2.35%, for BPA 10 ng/mL and 1 µg/mL, respectively) (Figure 2A), indicating that concentrations found in humans as a result of environmental exposure may affect aging of vascular endothelial cells.

A decline in cellular proliferation after prolonged culture is a common feature of primary cells associated with G0 arrest (Hwang et al. 2009), and in HUVEC this is accompanied by increased spontaneous apoptosis and polyploidization (Wagner et al. 2001). Our results clearly indicate significant decreases in proliferation capacity between passages 12 and 19 (Figure 2B). This is evident as significantly lower fluorescence emission at passage 19 for all growth conditions, which indicates decreased numbers of viable cells. Relevantly, this reduction in viable cells is more aggravated in BPA treated cells, showing that prolonged BPA exposure enhances this aging related phenotype.

Cellular morphology and cytoskeleton organization was compared between non-senescent (passage 6) and senescent cells (passage 19). As aging cells cease to divide, there is the emergence of several distinctive changes in morphology, motility and mechanical strength along with cytoskeleton alterations, such as an increase in microtubules (Hwang et al. 2009; Wang and Gundersen 1984). Immunodetection of α-tubulin clearly revealed a distinct morphology between young and aging HUVEC (Figure 2C). Senescent cells show augmented surface area, enlarged nuclear size, stellate outlines, and

microtubules enrichment, as previously described (van der Loo et al. 1998). Even though BPA can directly target tubulin (George et al. 2008) and disturb mitotic cytoskeleton on actively dividing HUVEC (Ribeiro-Varandas et al. 2013), it does not affect cytoskeleton organization on aging HUVEC. Although our cytological results did not identify alterations in cell morphology associated with BPA exposure, BPA induced effects on cell viability are supported by evident decreases in cell density at the later passage (Figure 2C).

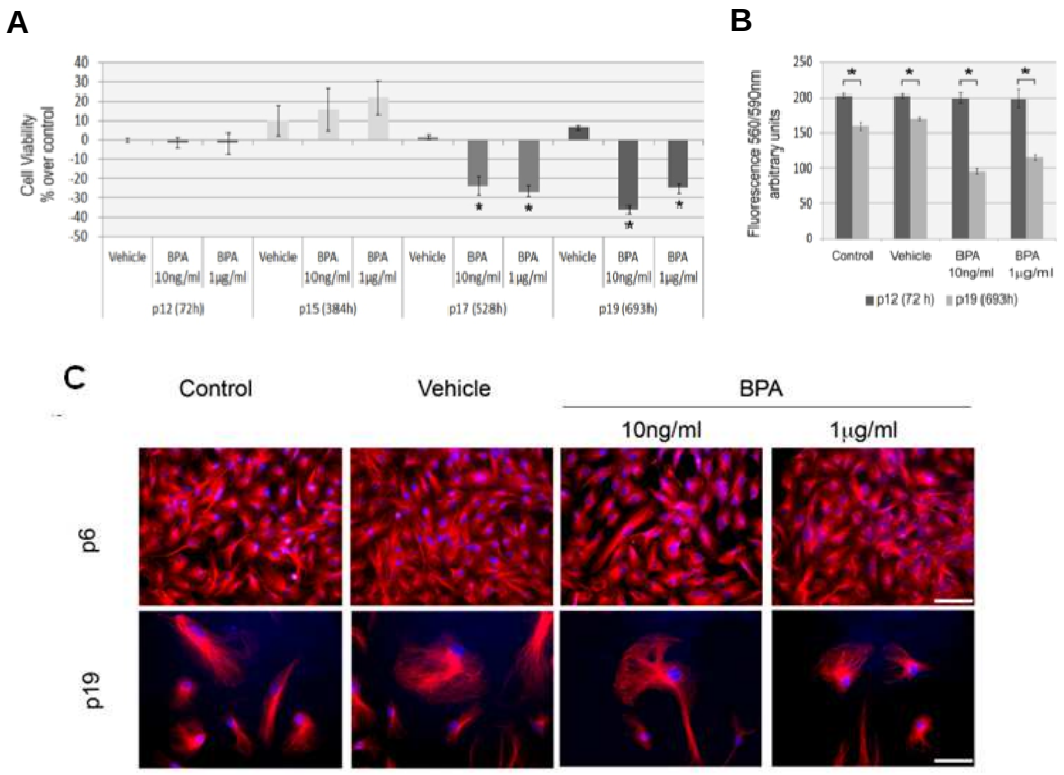


Figure 2 - Evaluation of BPA exposure effects on cellular viability and morphology on aging HUVEC cells. (**A**) Variation in cell viability after 72 h exposure (passage 12, p12), 384 h (passage 15, p15), 528 h (passage 17, p17) and 693 h (passage 19, p19) of exposure to vehicle, BPA 10 ng/mL and BPA 1 µg/mL. Results are presented as percentage of variation in relation to equivalent cells maintained in standard medium (control). Student's *t* test ***** $p < 0.01$ in relation to control; (**B**) Comparison of fluorescence intensity at 590 nm (excitation wavelength, 560 nm) between passage 12 and 19 for all culture conditions assayed. Results are presented as mean ± standard deviation. Student's *t* test ***** $p < 0.001$. Data in (**A**) and (**B**) result from cultivation and viability procedures performed simultaneously for all growth conditions; the same seeding density was used in all passages and growth conditions; (**C**) Cell morphology analysis after in situ detection of α-tubulin (red) and DNA DAPI staining (blue) in young proliferative HUVEC (P6, upper panel) and aging HUVEC (P19, lower panels). Young proliferative HUVEC were exposed to either BPA concentration or vehicle for 72 h at passage 6; aging cells were analyzed at passage 19 after continuous exposure from passage 12. Scale bars = 50 µm.

4.4.3 Continuous BPA exposure induces differential gene expression in aging HUVEC.

Stress-induced senescence is associated with modifications in gene expression profiles (Dumont et al. 2002; Toussaint et al. 2000). In order to analyze the effects of 1 µg/mL BPA short and continuous exposure, qRT-PCR was utilized to evaluate the expression levels of seven genes on young (p6) and aging (p12 and p19) HUVEC. Four genes for which transcription is altered in replicative senescence were selected, namely two major extracellular matrix proteins coding genes, osteonectin (*SPARC*) and fibronectin (*FN1*), and two regulators of cell fate, *p21* and *FOS*, (Debacq-Chainiaux et al. 2008; Wagner et al. 2001). The apoptotic gene *bcl-xL* was also evaluated, since BPA exposure has been associated to alterations of transcriptional expression of apoptotic related genes in several cell lines (Buterin et al. 2006; Naciff et al. 2010) and age-related increase in programmed cell death was shown to occur in HUVEC cells (Wagner et al. 2001). Lastly, given that nucleolus is one of the most frequent cellular structures associated with human premature aging syndromes, nucleolin gene (*NCL*) and 18S rRNA transcription levels were also analyzed (Puzianowska-Kuznicka and Kuznicki 2005). Short (72 h) exposure was evaluated in young cells (p6) and aging cells (p12) (Figure 3A) whereas continuous exposure was evaluated in aging cells maintained in the presence of BPA from passage 12 to passage 19 (p19) (Figure 3B). Results are presented in comparison to equivalent cells grown in medium supplemented with vehicle as mean log2 fold change ± standard deviation.

In young HUVEC (p6) 72 h exposure to 1 µg/mL BPA resulted, as we previously reported (Ribeiro-Varandas E. 2012), in a slight up-regulation of *NCL* gene (0.308 ± 0.340), which encodes for nucleolin a multifunctional protein associated ribosome biogenesis as well as to several other RNA regulatory mechanisms with proliferative and survival effects (Abdelmohsen et al. 2012). Inversely, in aging cells (p12) the same BPA exposure resulted in a significant decrease of *p21* (−0.41 ± 0.166) and *bcl-xL* (−0.49 ± 0.352) transcription levels. The down-regulation of both genes suggests a lower capacity of aging cells to respond to damage, as *p21* is a CDK inhibitor with a well established role in growth arrest in response to cell injury (de Camé Trécesson et al. 2011; Kim et al. 2001) and *bcl-xL* encodes for a transmembrane mitochondria protein with

anti-apoptotic function (Michels et al. 2013). Seventy-two-hour BPA exposure of aging cells does not result in immediate effects on cell viability (Figure 2A) as we have also reported for young HUVEC cells (Ribeiro-Varandas et al. 2013). However, continuous BPA exposure of aging HUVEC cells (693 h, p19) result in significant decrease in cell viability accompanied by significant down-regulation of the mRNA levels of both *FOS* (−0.884 ± 0.319) and 18S rDNA (−0.801 ± 0.588) *FOS* is a component of the AP-1 transcription factor that regulates distinct cellular processes including cell proliferation, death, survival and differentiation (Hess et al. 2004) and under stress conditions regulation of ribosome biosynthesis is one of the cellular strategies to preserve homeostasis (Boulon et al. 2010). The large variation (standard deviation) associated with the gene expression data is of particular interest, especially in regards to *SPARC* and *NLC* mRNA levels. Considering that the levels of variation between technical replicates were almost absent, this shows that BPA induces general and non-directional de-regulation of gene expression in senescent cells. This is further supported by the transcription analysis of L1 related sequences after continuous BPA exposure (693 h, p19) showing a decrease in L1-5' (−0.549 ± 0.368) and an increase in L1-3' (1.115 ± 0.15) (Figure 3C).

Overall, the gene transcription results obtained on senescent HUVEC continuously exposed to BPA clearly show that this chemical affects the aging process. HUVEC constitute an *in vitro* model in the investigation of atherosclerosis pathogenesis (Khaidakov et al. 2011), an intrinsically age-related disease in which vascular senescence play a critical role (Andreassi 2008). Relevantly, atherosclerosis has been related to both deficient endothelial cell turnover (Higashi et al. 2012) as well as increased cell death (Van Vre et al. 2012). Moreover several epidemiologic studies indicate that circulating BPA is correlated with coronary and carotid atherosclerosis (Lind and Lind 2011; Melzer et al. 2010; Melzer et al. 2012). Overall, our results indicate that BPA may play a role in atherosclerosis induction by decreasing proliferation capacity in association with transcriptional de-regulation.

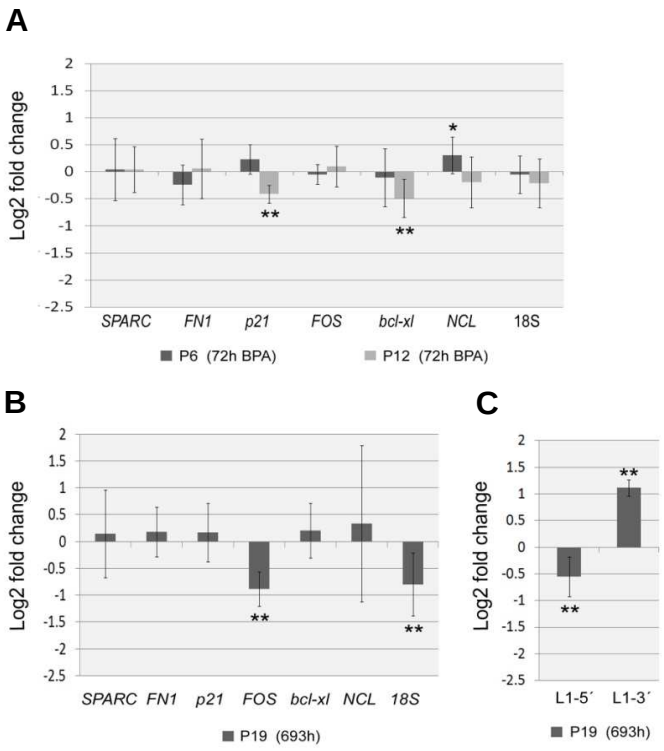


Figure 3 - Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) evaluation of osteonectin (*SPARC*), fibronectin (*FN1*), *p21*, *FOS*, *bcl-xl*, nucleolin (*NCL*) and *18S rRNA* transcription on (**A**) young (p6) and aging (p12) HUVEC cells after 72 h of 1 µg/mL BPA exposure and (**B**) aging HUVEC (p19) after prolonged (693 h) exposure to BPA 1 µg/mL; (**C**) L1-5' and L1-3' transcription levels on aging HUVEC after prolonged exposure to BPA 1 µg/mL (p19, 693 h). Results are shown as the mean log2 fold change ($2^{-\Delta\Delta Ct}$) ± standard deviation in relation to cells from equivalent passages maintained in medium supplemented with vehicle. Student's t test, **p < 0.01 and *p < 0.05.

4.5 Conclusion

For the past years, bisphenol A (BPA) has been implicated in the etiology of several human pathologies and its effects extensively studied. However, the current available information regarding BPA effects on aging processes is still scarce.

The present work demonstrates that BPA at low concentrations has the capacity to affect global transcription levels in both HT29 and HUVEC. Furthermore, we show for the first time that continuous exposure to BPA interferes with gene expression and severely decreases cellular viability in

aging vascular endothelial cells. These results support the correlation between BPA and atherosclerosis related diseases reported in distinct epidemiological studies and increasing concerns regarding the adverse effects of BPA exposure on human health.

4.6 References

Abdelmohsen K, Gorospe M. 2012. Rna-binding protein nucleolin in disease. RNA Biol 9:799-808.

Andreassi MG. 2008. DNA damage, vascular senescence and atherosclerosis. J Mol Med (Berl) 86:1033-1043.

Aporntewan C, Phokaew C, Piriyapongsa J, Ngamphiw C, Ittiwut C, Tongsima S, et al. 2011. Hypomethylation of intragenic line-1 represses transcription in cancer cells through ago2. PLoS One 6:e17934.

Astrof S, Hynes RO. 2009. Fibronectins in vascular morphogenesis. Angiogenesis 12:165-175.

Ballesteros-Gomez A, Rubio S, Perez-Bendito D. 2009. Analytical methods for the determination of bisphenol a in food. J Chromatogr A 1216:449-469.

Bar-On O, Shapira M, Hershko DD. 2007. Differential effects of doxorubicin treatment on cell cycle arrest and skp2 expression in breast cancer cells. Anticancer Drugs 18:1113-1121.

Boehme K, Simon S, Mueller SO. 2009. Gene expression profiling in ishikawa cells: A fingerprint for estrogen active compounds. Toxicology and Applied Pharmacology 236:85-96.

Boulon S, Westman BJ, Hutten S, Boisvert FM, Lamond AI. 2010. The nucleolus under stress. Mol Cell 40:216-227.

Boyd-Kirkup JD, Green CD, Wu G, Wang D, Han JD. 2013. Epigenomics and the regulation of aging. Epigenomics 5:205-227.

Bradshaw AD. 2012. Diverse biological functions of the sparc family of proteins. Int J Biochem Cell Biol 44:480-488.

Bredhult C, Sahlin L, Olovsson M. 2009. Gene expression analysis of human endometrial endothelial cells exposed to bisphenol a. Reprod Toxicol 28:18-25.

Buterin T, Koch C, Naegeli H. 2006. Convergent transcriptional profiles induced by endogenous estrogen and distinct xenoestrogens in breast cancer cells. Carcinogenesis 27:1567-1578.

D'Aquila P, Rose G, Bellizzi D, Passarino G. 2013. Epigenetics and aging. Maturitas 74:130-136.

de Carné Trécesson S, Guillemin Y, Belanger A, Bernard AC, Preisser L, Ravon E, et al. 2011. Escape from p21-mediated oncogene-induced senescence leads to cell dedifferentiation and dependence on anti-apoptotic bcl-xl and mcl1 proteins. J Biol Chem 286:12825-12838.

Debacq-Chainiaux F, Pascal T, Boilan E, Bastin C, Bauwens E, Toussaint O. 2008. Screening of senescence-associated genes with specific DNA array reveals the role of igfbp-3 in premature senescence of human diploid fibroblasts. Free Radic Biol Med 44:1817-1832.

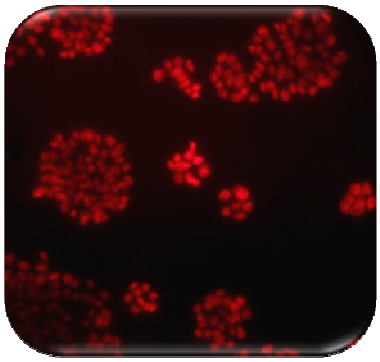
Doherty LF, Bromer JG, Zhou Y, Aldad TS, Taylor HS. 2010. In utero exposure to diethylstilbestrol (des) or bisphenol-a (bpa) increases ezh2 expression in the mammary gland: An epigenetic mechanism linking endocrine disruptors to breast cancer. Horm Cancer 1:146-155.

Dolinoy DC, Huang D, Jirtle RL. 2007. Maternal nutrient supplementation counteracts bisphenol a-induced DNA hypomethylation in early development. Proceedings of

the National Academy of Sciences of the United States of America 104:13056-13061.
Dong S, Terasaka S, Kiyama R. 2011. Bisphenol a induces a rapid activation of erk1/2 through gpr30 in human breast cancer cells. Environ Pollut 159:212-218.
Dumont P, Chainiaux F, Eliaers F, Petropoulou C, Remacle J, Koch-Brandt C, et al. 2002. Overexpression of apolipoprotein j in human fibroblasts protects against cytotoxicity and premature senescence induced by ethanol and tert-butylhydroperoxide. Cell Stress Chaperones 7:23-35.
Fernandez SV, Huang Y, Snider KE, Zhou Y, Pogash TJ, Russo J. 2012. Expression and DNA methylation changes in human breast epithelial cells after bisphenol a exposure. Int J Oncol 41:369-377.
George O, Bryant BK, Chinnasamy R, Corona C, Arterburn JB, Shuster CB. 2008. Bisphenol a directly targets tubulin to disrupt spindle organization in embryonic and somatic cells. ACS Chem Biol 3:167-179.
Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: The next generation. Cell 144:646-674.
He Y, Miao M, Wu C, Yuan W, Gao E, Zhou Z, et al. 2009. Occupational exposure levels of bisphenol a among chinese workers. J Occup Health 51:432-436.
Hess J, Angel P, Schorpp-Kistner M. 2004. Ap-1 subunits: Quarrel and harmony among siblings. J Cell Sci 117:5965-5973.
Higashi Y, Sukhanov S, Anwar A, Shai SY, Delafontaine P. 2012. Aging, atherosclerosis, and igf-1. J Gerontol A Biol Sci Med Sci 67:626-639.
Hon G, Wang W, Ren B. 2009. Discovery and annotation of functional chromatin signatures in the human genome. PLoS Comput Biol 5:e1000566.
Hwang ES, Yoon G, Kang HT. 2009. A comparative analysis of the cell biology of senescence and aging. Cell Mol Life Sci 66:2503-2524.
Khaidakov M, Wang X, Mehta JL. 2011. Potential involvement of lox-1 in functional consequences of endothelial senescence. PLoS One 6:e20964.
Kim JS, Lee S, Lee T, Lee YW, Trepel JB. 2001. Transcriptional activation of p21(waf1/cip1) by apicidin, a novel histone deacetylase inhibitor. Biochem Biophys Res Commun 281:866-871.
Li D, Zhou Z, Qing D, He Y, Wu T, Miao M, et al. 2010. Occupational exposure to bisphenol-a (bpa) and the risk of self-reported male sexual dysfunction. Hum Reprod 25:519-527.
Lind PM, Lind L. 2011. Circulating levels of bisphenol a and phthalates are related to carotid atherosclerosis in the elderly. Atherosclerosis 218:207-213.
Melzer D, Rice NE, Lewis C, Henley WE, Galloway TS. 2010. Association of urinary bisphenol a concentration with heart disease: Evidence from nhanes 2003/06. PLoS One 5:e8673.
Melzer D, Gates P, Osborne NJ, Henley WE, Cipelli R, Young A, et al. 2012. Urinary bisphenol a concentration and angiography-defined coronary artery stenosis. PLoS One 7:e43378.
Mezcua M, Martinez-Uroz MA, Gomez-Ramos MM, Gomez MJ, Navas JM, Fernandez-Alba AR. 2012. Analysis of synthetic endocrine-disrupting chemicals in food: A review. Talanta 100:90-106.
Michels J, Kepp O, Senovilla L, Lissa D, Castedo M, Kroemer G, et al. 2013. Functions of bcl-x l at the interface between cell death and metabolism. Int J Cell Biol 2013:705294.
Naciff JM, Khambatta ZS, Reichling TD, Carr GJ, Tiesman JP, Singleton DW, et al. 2010. The genomic response of ishikawa cells to bisphenol a exposure is dose- and time-dependent. Toxicology 270:137-149.
Ostertag EM, Kazazian HH, Jr. 2001. Biology of mammalian l1 retrotransposons. Annu Rev Genet 35:501-538.
Pankov R, Yamada KM. 2002. Fibronectin at a glance. J Cell Sci 115:3861-3863.
Ptak A, Wrobel A, Gregoraszczuk EL. 2011. Effect of bisphenol-a on the expression of selected genes involved in cell cycle and apoptosis in the ovcar-3 cell line. Toxicol Lett 202:30-35.

Pupo M, Pisano A, Lappano R, Santolla MF, De Francesco EM, Abonante S, et al. 2012. Bisphenol a induces gene expression changes and proliferative effects through gper in breast cancer cells and cancer-associated fibroblasts. Environ Health Perspect 120:1177-1182.
Puzianowska-Kuznicka M, Kuznicki J. 2005. Genetic alterations in accelerated ageing syndromes. Do they play a role in natural ageing? Int J Biochem Cell Biol 37:947-960.
Qin XY, Fukuda T, Yang L, Zaha H, Akanuma H, Zeng Q, et al. 2012. Effects of bisphenol a exposure on the proliferation and senescence of normal human mammary epithelial cells. Cancer Biol Ther 13:296-306.
Ribeiro-Varandas E, Viegas W, Pereira HS, Delgado M. 2013. Bisphenol a at concentrations found in human serum induces aneugenic effects in endothelial cells. Mutat Res 751:27-33.
Ribeiro-Varandas E. SPH, Monteiro S., Boavida Ferreira R., Neves E., Brito L., Viegas W., Delgado M. The environmental pollutant bisphenol a interferes with nucleolar structure. In: Proceedings of the International Conference on Biomedical Engineering and Biotechnology, 2012. Macau, pp.1811-1814.
Rivera LB, Bradshaw AD, Brekken RA. 2011. The regulatory function of sparc in vascular biology. Cell Mol Life Sci 68:3165-3173.
Rubin BS. 2011. Bisphenol a: An endocrine disruptor with widespread exposure and multiple effects. J Steroid Biochem Mol Biol 127:27-34.
Singh S, Li SS. 2012. Epigenetic effects of environmental chemicals bisphenol a and phthalates. Int J Mol Sci 13:10143-10153.
Toussaint O, Medrano EE, von Zglinicki T. 2000. Cellular and molecular mechanisms of stress-induced premature senescence (sips) of human diploid fibroblasts and melanocytes. Exp Gerontol 35:927-945.
van der Loo B, Fenton MJ, Erusalimsky JD. 1998. Cytochemical detection of a senescence-associated beta-galactosidase in endothelial and smooth muscle cells from human and rabbit blood vessels. Exp Cell Res 241:309-315.
Van Vre EA, Ait-Oufella H, Tedgui A, Mallat Z. 2012. Apoptotic cell death and efferocytosis in atherosclerosis. Arterioscler Thromb Vasc Biol 32:887-893.
Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgartten FJ, Schoenfelder G. 2010. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol a. Environ Health Perspect 118:1055-1070.
Varier RA, Timmers HT. 2011. Histone lysine methylation and demethylation pathways in cancer. Biochim Biophys Acta 1815:75-89.
Wagner M, Hampel B, Bernhard D, Hala M, Zwerschke W, Jansen-Durr P. 2001. Replicative senescence of human endothelial cells in vitro involves g1 arrest, polyploidization and senescence-associated apoptosis. Exp Gerontol 36:1327-1347.
Wang E, Gundersen D. 1984. Increased organization of cytoskeleton accompanying the aging of human fibroblasts in vitro. Exp Cell Res 154:191-202.
Yaoi T, Itoh K, Nakamura K, Ogi H, Fujiwara Y, Fushiki S. 2008. Genome-wide analysis of epigenomic alterations in fetal mouse forebrain after exposure to low doses of bisphenol a. Biochemical and Biophysical Research Communications 376:563-567.

5 Bisphenol A at the reference level counteracts Doxorubicin transcriptional effects on cancer related genes in HT29 cells



Margarida Delgado and **Edna Ribeiro-Varandas**. Bisphenol A at the reference level counteracts Doxorubicin transcriptional effects on cancer related genes in HT29 cells. Submitted to Environmental Health Perspectives, manuscript ID: 14-08401-ART, 2014. Both authors contributed equally.

5.1 Abstract

Background: Human exposure to Bisphenol A (BPA) results mainly from ingestion of food and beverages. Information regarding BPA effects on colon cancer, one of the major causes of death in developed countries, is still scarce. Likewise, little is known about BPA drug interactions although its potential role in doxorubicin (DOX) chemoresistance has been suggested.

Objective: This study aims to assess potential interactions between BPA and DOX on HT29 colon cancer cells.

Methods: HT29 cell response was evaluated after exposure to BPA, DOX, or co-exposure to both chemicals. Transcription levels of several cancer-associated genes (*c-fos*, *AURKA*, *p21*, *bcl-xl* and *CLU*) were quantified by qRT-PCR and cell viability analyzed through resazurin assay. Apotosis induction and mitotic disruption were evaluated cytologicaly by TUNEL assay and DAPI staining, respectively.

Results: Gene expression analysis shows that BPA exposure induces slight up-regulation exclusively of *bcl-xl* without affecting cell viability. Sub-therapeutic DOX concentration (40 nM) results in highly altered *c-fos*, *bcl-xl*, and *CLU* transcript levels, and this is not affected by co-exposure with BPA. In contrast, therapeutic DOX concentration (4 µM) induced down-regulation of *c-fos* and up-regulation of *AURKA*, *p21* and *CLU* is counteracted by BPA, evident as similar transcript levels in co-exposed cells to those of control. Co-exposure with BPA slightly decreases apoptosis in relation to DOX 4 µM alone without affecting DOX-induced loss of cell viability.

Conclusions: These results emphasize the necessity of a better understanding of BPA interactions particularly with chemotherapeutic agents in the context of risk assessment.

5.2 Introduction

Bisphenol A (BPA) is a plastic monomer and plasticizer used in a wide range of consumer products including food and drink containers and oral intake is accepted as being the main route of human exposure (Geens et al. 2012).

Although BPA is considered a weak environment estrogen due to its low affinity to classical nuclear estrogen receptors (ER) (Pennie et al. 1998), several findings reveal that BPA prompt very distinct responses through a variety of signaling pathways (Rubin 2011). BPA has been intensively studied in regard to carcinogenesis in hormone responsive organs (Soto and Sonnenschein 2010). However, an aspect poorly investigated is BPA potential interactions with chemotherapeutic drugs although there are some evidences of its counteracting effects. In breast cancer cells the anti-proliferative effects of distinct commonly used chemotherapeutic drugs namely doxorubicin (DOX), vinblastine, and cisplatin are antagonized by BPA and associated with increased levels of anti-apoptotic proteins (LaPensee et al. 2009; 2010).

On the other hand, colon cancer is one of the most common causes of death in industrialized countries (WHO 2014). Relevantly, colon cancer incidence is rising in developing countries reflecting dietary changes (Center et al. 2009) and likewise BPA human exposure is also increasing (Nahar et al. 2012). Even so, research on BPA effects on the intestinal tract has received little attention and is limited to few studies. BPA was shown to increase tight junction sealing in epithelial colon cells in rats (Braniste et al. 2010) and impair the estradiol inhibitory effect on proliferation of colon adenocarcinoma cells DLD-1 (Bolli et al. 2010).

In the present study we aimed to evaluate effects of BPA on colon adenocarcinoma cell line HT29 and its interaction with DOX. Distinct cellular effects have been associated with DOX depending on concentration. Commonly used therapeutic dosages induce cell cycle arrest and apoptosis while lower concentrations are associated with mitotic disruption and senescence-like phenotypes (Eom et al. 2005; Park et al. 2007). Considering this, two DOX concentrations were assayed: a sub-therapeutic dosage for which interaction with BPA was previously reported (LaPensee et al. 2009) and a 100 fold higher concentration corresponding to a therapeutic level (Greene et al. 1983). Although human exposure to BPA is accepted as generalized the level of exposure is a matter of intense debate. In 1993 the U.S. Environmental Protection indicated as Reference Dose for Chronic Oral Exposure (RfD) for BPA 50 μg/kg body weight/day (EPA 1993) and in 2006 the European Food

Safety Authority set the same value as BPA Tolerable Daily Intake (TDI) (EFSA 2006). Human exposure to BPA trough diet, estimated on migration values, point to a range of 0.4–4.2 μg/kg body weight/day (Geens et al. 2012) corresponding to approximately 100 to 10 fold less than the TDI. Such estimations are compatible with BPA levels detected in urine, however there is a discrepancy with biomonitoring studies that indicate the presence of unmetabolized BPA in human blood/serum in the range of 2–13 nmol/l (0.5–3 ng/ml) (Vandenberg et al. 2010). Human studies in a limited number of individuals subjected to BPA oral exposure reveal however much lower levels of circulating BPA (Völkel et al. 2002; 2005). On the other hand, studies in non-human primates point to the need of oral dosages higher than the TDI to reach blood levels identical to those found in humans (Fisher et al. 2011; Taylor et al. 2011). Another point of debate in risk assessment is the fact that BPA undergoes extensive gut and liver glucuronidation and consequently only a small fraction of unmetabolized bioactive BPA enters the blood stream. BPA pharmacokinetic models are based in a rapid BPA glucuronidation and urinary clearance (Edginton and Ritter 2009; Fisher et al. 2011) but do not take into consideration potential local BPA deconjugation at particular tissues. β-glucuronidase-mediated BPA deconjugation in placenta and fetal tissues has been suggested to contribute to considerable fetus exposure to free BPA (Ginsberg and Rice 2009). Significantly, extracellular β-glucuronidase levels are increased in most solid tumors microenvironment due to the inflammatory process and lysosomal release (Tranoy-Opalinski et al. 2014). In recent years, therapeutic approaches that specifically target tumors have taken advantage of this characteristic through the development of glucuronide prodrugs that are activated in the β-glucuronidase-rich microenvironment (Tranoy-Opalinski et al. 2014). In contrast with other internal organs, for which exposure depends on circulating BPA, in the digestive tract additional contact with ingested BPA prior to its conjugation can contribute to the overall cell exposure. Considering the high level of uncertainty regarding BPA burden in intestinal tissues and particularly in tumors we choose to evaluate BPA effects at a concentration corresponding to the established guidance values and considered safe in terms of human exposure. Moreover, our previous results show that colon

adenocarcinoma cell line HT29 is not considerably affected by single exposure to this BPA concentration in terms of cell proliferation and transcription levels of cell cycle related genes (Ribeiro-Varandas et al. 2013).

For gene transcription analysis five distinct genes were selected which are generally recognized as key players in cancer biology. Besides the anti-apoptotic gene *bcl-xl*, for which increased expression was shown to occurs due to BPA/DOX interaction (LaPensee et al. 2009), *p21, AURKA, c-fos* and *CLU* were also analyzed. *p21* gene product is a cyclin-dependent kinase inhibitor tightly connected with cell cycle progression and cancer outcome (Warfel and El-Deiry 2013). *AURKA* encodes for Aurora A, a mitotic key regulatory protein associated to cancer aggressiveness (Nikonova et al. 2013). The *c-fos* encoded protein is a component of the activator protein-1 (AP-1) transcription factor required for accurate regulation of several genes associated with cell proliferation, differentiation, apoptosis and oncogenic transformation (Durchdewald et al. 2009). *CLU* encodes for clusterin a multifunctional protein that has been implicated both in promotion of carcinogeneis as well as in tumor suppression (Mazzarelli et al. 2009; Pucci et al. 2009). Additionally to gene transcription analysis, cytological evaluation of apoptosis and mitotic disruption as well as cell viability assays were also carried for assessment of potential BPA/DOX interactions.

5.3 Materials and Methods

Cell culture and reagents.

HT29 cells were purchased from ECACC and cultivated in 75cm2 flasks with RPMI media containing GlutaMAX™ I, 25 mM HEPES (Invitrogen), supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. Cells cultures were maintained in a 5% (v/v) CO_2 humidified atmosphere at 37°C. After subculture cells were allowed to stabilize for 24 h in standard growth medium before treatments.

Drugs and treatments.

Bisphenol A (Sigma) was freshly diluted in ethanol and added to the culture media to the final concentration of 4.4 µM (1 µg/ml) that corresponds to the TDI considering an average body weight of 70 Kg and daily consumption of 3 liters of preformed water. Doxorubicin (AppliChem) was dissolved in water and added to the culture medium to final concentration of 40 nM (25 ng/ml) or 4 µM (2.5 µg/ml), the later corresponding to free DOX concentration in blood in clinical cancer chemotherapy (Greene et al. 1983). For the combined BPA/DOX exposures cells were pre-exposed to BPA for 24 h followed by additional 24 h of simultaneous exposure to BPA and DOX. Correspondingly, for single drug exposures cells were incubated with BPA for 48 h after the 24 h stabilization period whereas for DOX standard medium was substituted for medium with DOX 48 h after subculture and maintained. For evaluation of cell recovery capacity after treatments cells were cultivated for additional 72 h in standard culture medium. Controls were performed for all experiments using cells grown in standard culture medium or in medium supplemented with ethanol 170 µM (vehicle concentration for BPA).

Real-time PCR

Transcriptional analysis was performed by quantitative real-time RT-PCR (qRT-PCR) for *c-fos*, *AURKA*, *p21, bcl-xl* and *CLU* genes as well as control genes *GAPDH* and *β-actin*, using the specific primers listed in Table 1. Total RNA was extracted with the RNAqueous Kit (Invitrogen) following manufacturers´ instructions and 3 µg utilized for RNase free DNase digestion (RQ1 RNase free DNase, Promega). cDNA synthesis was completed with random primers (DYNAmo cDNA syntesis Kit, Thermo Scientific) and utilized for qRT-PCR with SsoFast Eva Green Supermix (BioRad) utilizing the following conditions: 95 ◦C for 3 min, 35 cycles (95 ◦C for 30 s, 55 ◦C for 30 s, 72 ◦C for 40 s), and 72 ◦C for 5 min. To ensure that genomic DNA was completely absent prior to cDNA synthesis, PCRs were performed with 18S rDNA primers and 250 ng of DNase digested RNA. Control PCRs were also performed for all primer combinations without template. Denaturation curves were observed to ensure correct amplification products. Since no significant differences were detected between

the two reference genes, threshold cycles (Ct) of the target genes were equilibrated with the mean Ct of *GAPDH* and *β-actin* genes to calculate ΔCt (ΔCt = Ct target – mean Ct *GAPDH:β-actin*). Gene expression levels were analyzed by calculating ΔΔCt (ΔΔCt = ΔCt treatment – mean ΔCt control). Results are presented as log2 of the mean fold change ($2^{-\Delta\Delta Ct}$) ± standard deviation.

Table 1 Primers used for qRT-PCR.

Gene	Accession nº	Forward primer (5´→3´)	Reverse primer (5´→3´)	Product size (bp)
AURKA	NM_003600	GCTGGAGAGCTTAAAATTGCAG	TTTTGTAGGTCTCTTGGTATGTG	220
c-fos	NM_005252	AGGAGAATCCGAAGGGAAAG	CAAGGGAAGCCACAGACATC	247
p21	NM_000389	CTGGAGACTCTCAGGGTCGAA	CCAGGACTGCAGGCTTCCT	123
bcl-xl	Z23115.1	TTACCTGAATGACCACCTA	ATTTCCGACTGAAGAGTGA	185
CLU	NM_001831	GGATGAAGGACCAGTGTGACAAG	CAGCGACCTGGAGGGATTC	114
GAPDH	NM_002046	GAGTCAACGGATTTGGTCGTA	GCAGAGATGATGACCCTTTTG	245
β-Actin	NM_001101	GGTCATCTTCTCGCGGTTGGCCTTGGGGT	CCCCAGGCACCAGGGCGTGAT	230

[a] GenBank accession numbers (National Center for Biotechnology).

Cytotoxicity assay.

Cell viability was evaluated by resazurin assay (CellTiter-Blue, Promega) following manufacturer's instructions and fluorescence emission measured with a microplate reader (Synergy HT, BioTek). Cells were plated on 96-well dishes at a density of 3.2×10^4 cells/well.

TUNEL assay.

For cell death assessment, cells were grown over glass coverslips coated with 0.2% (v/v) gelatin (Sigma) and after treatments fixed in 4% (p/v) formaldehyde in PBS for 10 min at room temperature and subsequently TUNEL assay (Roche) was performed accordingly to manufacturers´ instructions followed by DAPI staining.

Cytology analysis of mitosis.

Cells were cultivated on Petri dishes as above. After treatments cells were fixed in 4% (p/v) formaldehyde in PBS for 10 min at room temperature and DAPI stained for evaluation mitotic index and mitotic anomalies.

Data analysis.

All experiments were repeated at least three times with at least three replicates per experiment. For all parameters analyzed no significant differences were obtained between cells grown in standard medium or in ethanol-supplemented medium, the pooled results are and indicated as control. χ^2 test was used for analysis of relative occurrence of distinct types of mitotic anomalies. Student´s t test was used for analysis of cellular viability, frequency of apoptotic bodies, mitotic index and qRT-PCR gene transcription analysis. Additionally, for qRT-PCR data only differences in transcription levels higher than 1 for the mean log2 fold change (corresponding to a difference in one PCR cycle and a value of 2 for the Fold Change) were considered for statistical analysis.

5.4 Results

5.4.1 BPA at reference level increases transcription of *bcl-xl*.

Transcription levels were analyzed for cancer related genes *c-fos*, *AURKA*, *p21*, *bcl-xl* and *CLU* in HT29 cells exposed to BPA at a concentration corresponding to the TDI level (4.4 µM) during 48 h. Figure 1A shows that BPA exposure did not alter mRNA levels of *c-fos*, *AURKA*, *p21* and *CLU* genes. Conversely, after exposure to BPA an up-regulation was detected for the antiapototic gene *bcl-xl* corresponding to transcript duplication in relation to control (log2 fold change 1.1 ± 0.68).

5.4.2 BPA does not affect gene expression alterations induced by low DOX concentration.

HT29 cells exposure to DOX alone at sub-therapeutic concentration (40 nM) for 24 h revealed a clear effect on transcription levels of distinct genes. Although *AURKA* and *p21* genes were not affected by DOX, the levels of *c-fos*, *bcl-xl* and *CLU* were significantly altered (Figure 1B). *CLU* transcript level was strongly increased (log2 fold change 2.84 ± 0.12) by DOX 40 nM whereas *c-fos* showed a severe down-regulation (log2 fold change -4.73 ± 0.35) that was also patent for *bcl-xl* although at a minor extent (log2 fold change -1.96 ± 0.32).

Combined exposure to BPA 4.4 µM and DOX 40 nM, corresponding to 24 h pre-exposure to BPA alone followed by additional 24 h of simultaneous exposure to BPA and DOX, resulted in altered gene transcription patterns in relation to control but similar to that obtained for DOX 40 nM alone (Figure 1B).

5.4.3 Gene expression alterations induced by DOX at therapeutic concentration are reverted by BPA.

Exposure of HT29 cells to DOX therapeutic concentration (4 µM) for 24 h resulted in a significant variation in relation to control for four of the five genes analyzed; only *bcl-xl* gene transcription was not noticeably altered (Figure 1C). *c-fos* was significantly down-regulated (log2 fold change 3.04 ± 0.40), whereas *AURKA*, *p21* and *CLU* were up-regulated with a particularly prominent increase for *CLU* gene transcripts (Log2 fold change of 1.53 ± 0.36, 2.05 ± 1.09 and 5.99 ± 0.18 for *AURKA*, *p21* and *CLU*, respectively). Strikingly, pre-exposure to BPA (4 µM) for 24 h and subsequent 24 h combined exposure to BPA and DOX at therapeutic concentration (4 µM) resulted in significant changes in transcription for all genes analyzed in relation to DOX 4 µM alone (Figure 1C). A reversal of the effects induced by DOX therapeutic concentration was observed with BPA combined exposure. The four genes (*c-fos*, *AURKA*, *p21* and *CLU*) for which transcription was altered by DOX 4 µM, showed transcription levels identical to those observed in the control after exposure to BPA/DOX 4 µM. Only for *bcl-xl* no major variation was observed between DOX 4 µM alone and BPA/DOX 4 µM or between both treatments and control (log2 fold change -0.7 ± 0.51 and 0.51 ± 0.29 for DOX 4 µM and BPA/DOX 4 µM, respectively).

5.4.4 DOX sub-therapeutic and therapeutic concentrations have differential effects on gene transcription.

Comparative analyses of DOX exposure alone on gene transcription levels revealed significant differences in the effects of both DOX concentrations assayed for all genes analyzed ($p<0.001$ for each gene between DOX 40 nM and DOX 4 µM) (Figure 1B and C). For the lower DOX concentration (40 nM) *bcl-xl* transcription was significantly reduced in relation to control but no relevant alteration was detected for the higher concentration (4 µM). Contrarily, both

AURKA and *p21* were significantly up-regulated for the higher concentration DOX with no considerable effect for the lower concentration. *c-fos* transcript level was reduced in both concentrations but much more strongly for DOX 40 nM. Both DOX concentrations lead to an increase in *CLU* transcription level however much more pronounced for DOX 4 µM.

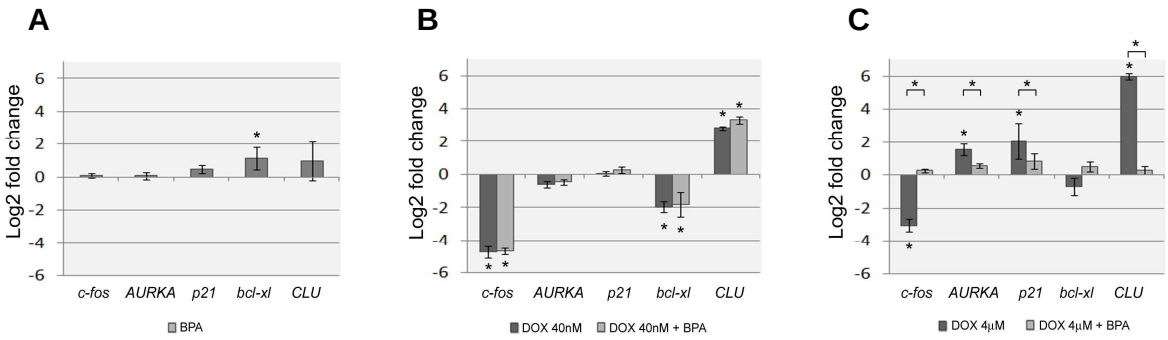


Figure 1 - BPA effect on HT29 gene transcription. *c-fos*, *AURKA*, *p21 bcl-xl* and *CLU* gene transcription analysis after exposure to (**A**) BPA 4.4 µM, (**B**) DOX 40 nM alone and in combination with BPA and (**C**) DOX 4 µM alone and in combination with BPA. Results are shown as average mean log 2 fold change ± standard deviation. * $p < 0.0001$ for Student´s t test for comparison between treatments and control or between distinct treatments (indicated by horizontal brackets).

5.4.5 BPA decreases DOX-induced apoptosis without immediate effects on cell viability.

Considering that DOX effect on apoposis is dependent on concentration and the differential results obtained between treatments for *bcl-xl* transcript levels, an evaluation of apoptosis was performed by TUNEL assay. TUNEL positive cells were virtually absent after exposure to BPA alone or to 40 nM DOX either alone or in combination with BPA. On the other hand, 4 µM DOX and BPA/DOX 4 µM resulted in the formation of typical TUNEL positive apoptotic bodies (Figure 2A). However comparison of apoptotic bodies frequency between 4 µM DOX and BPA/DOX 4 µM revealed a slightly but significant reduction associated with the BPA/DOX combined exposure (Figure 2B).

Effects on cell viability were accessed trough Resazurin assay immediately after treatments as well as after 72 h recovery in standard medium. No significant variation on cell viability was detected between treatments and control immediately after exposure (data not shown). The results obtained for all

treatments after recovery in drug free medium are presented in Figure 3 expressed as percentage in relation to control. No significant differences in cell viability were detected associated with exposure to BPA alone or to the lower DOX concentration (40 nM) either alone or combined with BPA. On the other hand a strong decrease in cell viability was detected for cells exposed to the higher DOX concentration (4 µM) either as single exposure or combination with BPA, with no significant difference between both treatments.

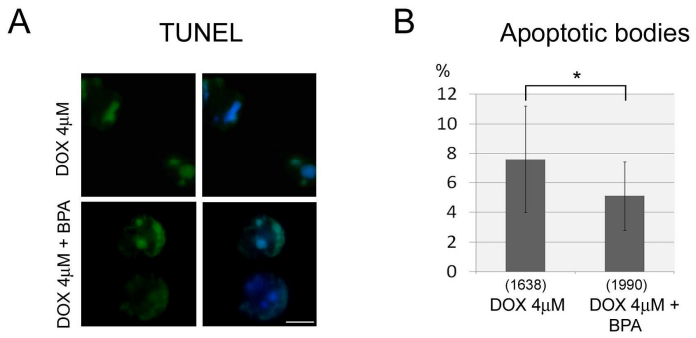


Figure 2 - BPA decreases DOX induced apoptotic bodies. **(A)** TUNEL positive nuclei (green) after exposure to DOX 4 µM alone and after BPA/DOX 4 µM combined exposure, merged images with DNA DAPI staining (blue) are shown on the right, bar = 5 µm. **(B)** Percentage of apoptotic bodies after exposure to DOX 4 µM and BPA/DOX 4 µM. The total number of cells analyzed is shown in brackets. * $p < 0.05$ for Student´s t test between treatments.

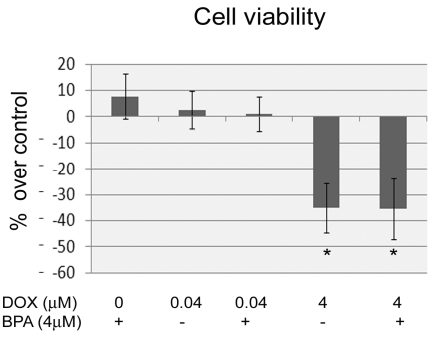


Figure 3 - DOX effect on cell viability is not affected by co-exposure with BPA. Cell viability after 72 h recovery in drug free medium following exposure to BPA, DOX (40 nM or 4 µM) and BPA/DOX (40 nM or 4 µM) co-exposure. Results are presented as percentage of variation over control, * $p < 0.01$ for Student´s t test in relation to control.

5.4.6 BPA does not affect mitotic disruption induced by DOX.

Mitotic disruption was also evaluated through cytological analysis after DNA DAPI staining immediately after treatments. The mitotic index was determined for all conditions assayed. Exposure to DOX 4 μM either alone or in combination with BPA resulted in cell cycle arrest reflected by the complete absence of mitotic cells (Figure 4A). On the other hand, no significant difference in the mitotic index in relation to control was detected for BPA 4.4 μM alone. Conversely, a reduction in the percentage of mitotic cells was observed after exposure to DOX 40 nM and identically for BPA/DOX 40 nM (Figure 4A). Quantification of abnormal mitotic cells revealed moreover an increase in relation to control associated with exposure to BPA, DOX 40 nM and BPA/DOX 40 nM. The mitotic abnormalities were further characterized accordingly with the phenotype observed as multipolar metaphases, anaphase bridges and lagging chromosomes (or chromosome fragments) (Figure 4B, i, ii and iii). Interestingly, the relative proportion of these types of mitotic anomalies was distinct between treatments (Figure 4B). For BPA exposure alone multipolar metaphases were predominant (79.4%), anaphase bridges were also frequent (20.6%) but lagging chromosomes were not detected. On the other hand, for exposure to DOX 40 nM, alone or in combination with BPA, anaphase bridges were prevalent (83.9% and 80.0%, for DOX 40 nM and BPA/DOX 40 nM, respectively), although some multipolar metaphases (12.9%, and 17.1%) and few chromosome fragments (3.2% and 2.9%) were also detected.

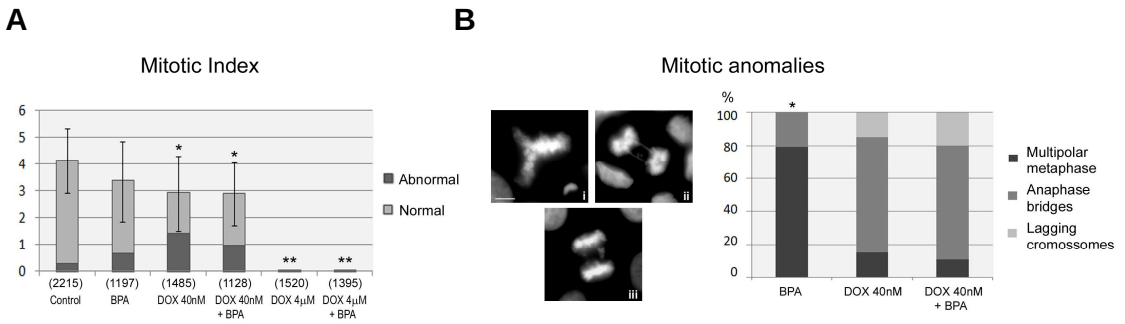


Figure 4 - DOX induced mitotic disruption is not affected by BPA. **(A)** Mitotic index and mitotic anomalies in control medium and after BPA, DOX 40 nM and DOX 4 µM exposure alone or BPA/DOX (40 nM or 4 µM) co-exposure. * $p < 0.01$ and ** $p < 0.001$ for Student´s t test in relation to control, total numbers of cells analyzed showed in brackets. **(B)** Quantification of distinct mitotic anomalies associated with DOX, BPA and combined BPA/DOX exposures. DAPI stained anomalous mitotic cells are shown on the left: (i) multipolar metaphase, (ii) anaphase bridges and (iii) lagging chromosomes, bar = 5 µm. Graphic on right shows the relative percentage of the different types of mitotic anomalies. At least 50 anomalous mitosis were analyzed for each treatment, * $p < 0.0001$ for χ^2 test for BPA exposure alone in relation to DOX 40 nM and BPA/DOX 40 nM.

5.5 Discussion

The present study shows that BPA at reference level (TDI) elicit very distinct cellular responses in HT29 colon adenocarcinoma cells when combined with DOX at low concentration (sub-therapeutic level) or when combined with DOX at high concentration (therapeutic level). Exposure to BPA alone does not alter cell viability but induces a slight up-regulation of the anti-apoptotic gene *bcl-xl*. It has been previously shown that BPA increases expression of anti-apoptotic genes in breast and ovarian cancer cells, although at lower concentrations and associated with increased cell proliferation (LaPensee et al. 2009; Ptak et al. 2011). On the other hand, we have demonstrated before that BPA at TDI level as well as at a lower concentration compatible with those found in humans do not alter cell proliferation of HT29 but also of primary Human Umbilical Vein Endothelial Cells (HUVEC) (Ribeiro-Varandas et al. 2013). Nonetheless, gene transcription analysis of cell cycle related genes revealed that HT29 are less sensible to BPA than HUVEC independently of BPA concentration, although both cell types are ERβ positive and HT29 additionally expresses the

membrane bound G protein-coupled estrogen receptor 1 (Ribeiro-Varandas et al. 2013). Therefore, BPA induction of *bcl-xl* in HT-29 cells suggests that this is a common feature of cellular response to BPA exposure independent of its positive effect on cell proliferation.

DOX triggers apoptosis at typical therapeutic doses but not at lower concentrations (Eom et al. 2005; Park et al. 2007). In a hepatocellular carcinoma it was demonstrated that DOX high concentration (20 μM) leads to apoptosis whereas DOX low concentration (100 nM) induces a senescence-like phenotype and mitotic catastrophe (Eom et al. 2005). It was also shown that over expression of *bcl-xl* does not block low dose DOX effects but impairs apoptosis induced by high concentration of DOX (Park et al. 2007). Here we show that sub-therapeutic DOX (40 nM) induces down-regulation of *bcl-xl* and an increase in mitotic anomalies in HT29 cells but not occurrence of apoptotic bodies with no effects on cell viability. A strong down-regulation of *c-fos* is also induced by DOX 40 nM concentration. *C-fos* is a member of the AP-1 transcription factor complex that was shown to promote *bcl-xl* transcription (Salameh et al. 2010). These observations indicate that also in colon adenocarcinoma HT29 cells DOX at low concentration induces mitotic disruption but not apoptosis.

On the other hand, the present results also show that exposure of HT29 to high DOX concentration (4 μM) results in cell viability decrease and apoptosis induction, without significant alteration in *bcl-xl* mRNA level. However, this DOX concentration induces marked transcriptional alterations in the other four genes analyzed. *C-fos* is down-regulated, as for DOX low concentration although to a lesser extent. Conversely, the mRNA levels of *AURKA* and *p21* are increased by DOX 4 μM what is compatible with the observed induction of cell cycle arrest. DOX at high concentrations is known to induce G2/M block through *p21* induction in several cell lines (Kim et al. 2009; Venkatakrishnan et al. 2008) what correlates with *AURKA* mRNA increase since *AURKA* transcription peaks at G2/M phase of the cell cycle (Tanaka et al. 2002). Cell sensitivity to DOX is correlated with the level of *p21* induction being higher for cells with functional *p53*, however it occurs also in HT29 cells that carry a *p53* mutation through a independent pathway (Ravizza et al. 2004).

The present work further demonstrates that the same BPA concentration, corresponding to the TDI level, has very distinct interaction outcomes with DOX at sub-therapeutic and therapeutic dosages. The HT29 response to DOX low concentration is not altered by co-exposure with BPA either at gene transcription level, cell viability or mitotic disruption. We have previously shown in HUVEC cells that BPA exposure alone leads to spindle anomalies and formation of multipolar metaphases (Ribeiro-Varandas et al. 2013). The present results show also a predominance of multipolar metaphases in HT29 cells exposed to BPA alone this effect is however masked by co-exposure with DOX 40 nM as the type of mitotic anomalies is identical to that of DOX 40 nM alone with predominance of anaphase bridges.

More importantly, the present results show that BPA at TDI level counteracts effects of DOX therapeutic dose on gene expression in colon adenocarcinoma cell line HT29 without short-term effects on cell viability. Although co-exposure with BPA is associated with a decrease in apoptotic cells, it does not alter the DOX 4 μM induced cell cycle arrest or loss of cell viability after drug removal. The slight reduction of apoptotic cells might not have a direct impact on overall cell viability and this effect of BPA/DOX 4 μM might be related with a small increment in the *bcl-xl* mRNA level in relation to DOX alone, although the difference in the transcription level is below the threshold established that corresponds to a 2-fold change. More puzzling is however that the DOX 4 μM induced cell arrest is not altered by combined exposure with BPA although transcription levels of both *p21* and *AURKA* are severely decreased in relation to DOX 4 μM alone. For the BPA/DOX combination the mRNA levels of both *p21* and *AURKA* are slightly above the control but this increase is below the 2-fold change threshold. If for DOX 4 μM alone the observed cell cycle arrest can be directly associated with *p21* induction, it is more difficult to understand that an identical result is obtained for the much lower level of *p21* detected after co-exposure to BPA/DOX and that this is also accompanied by a decrease in *AURKA* transcription in relation to DOX alone.

Significantly, the BPA counteracting effect of DOX 4 μM on gene expression is not limited to the cell cycle related genes *p21* and *AURKA* but also observed for the AP-1 transcription factor component *c-fos* as well as for *CLU*, which

encodes a multifunction protein. It is noteworthy that independently of DOX induced up- or down-regulation, the transcription levels of these four genes after combined exposure to BPA and DOX are similar to those of control. It can be argued that the transcription deregulation of components of transcription factors as *c-fos* may result in deregulation of other genes. It has been suggested that *c-fos* acts as transcriptional repressor of *CLU* (Jin and Howe 1999) and *c-fos* and *p21* levels have also shown to be inversely correlated in colonic epithelial cells associated with prolonged oxidative stress (Poehlmann et al. 2013). This raises the hypothesis that restoration to near control levels of *p21* and *CLU* mRNA levels in BPA/DOX 4 μM combination could result from the effect on *c-fos* transcription. However, a fundamental question remains unanswered: how BPA alters DOX induced deregulation of the transcription factors? The understanding of the mechanism underlying this interaction between BPA and DOX is out of the scope of this work but certainly a most important and complex problem. The mechanisms of action of BPA *per se* are not yet fully understood and although BPA is generally characterized as a xenoestrogen there is increasing evidences that can affect a variety of cellular pathways not necessarily mediated by classic estrogen receptors (Rubin 2011). Nonetheless, the present work demonstrates that BPA alters the transcriptional response to therapeutic DOX concentration of four genes for which there is vast literature establishing their key functions in cancer biology. As example it is worth noticing some of the more recent research regarding colon cancer. In colon cancer stem cells it was shown that siRNA knockdown of *c-fos* results in the up-regulation of other transcription factors, namely NANOG, OCT3/4, and SOX2 with important roles in maintaining cell pluripotency and self-renewal (Apostolou et al. 2013). In a distinct approach, an epidemiological study involving 386 stage II and III colon cancer patients revealed that disease free survival post surgical resection is positively related with high levels of *p21* and low levels of *AURKA* and that for stage III cancers with *AURKA* overexpression recurrence is potentiated by chemotherapy (Belt et al. 2012). On the other hand, *CLU* has received particular attention due to its implication in cancer progression, prognosis and chemoresistance, (Mazzarelli et al. 2009; Pucci et al. 2009). Interestingly, it was recently shown that ERβ signaling can indirectly increase *CLU* mRNA levels

through miRNA regulation (Edvardsson et al. 2013). Carcinogenesis, cancer progression, treatment response and disease outcome are undoubtedly dependent on gene expression patterns of malignant cells. The present results demonstrate that gene transcription response to DOX can be altered by BPA emphasizing the necessity to address effects of BPA interactions in terms of human health risk assessment.

5.6 Conclusion

Overall the data here presented reveals that BPA can alter the effect of DOX on transcript levels of crucial genes involved in cancer biology. Moreover, the ability of BPA to interact with DOX is dependent on DOX concentration and thus of the cellular processes affected by DOX with evident interaction effects restricted to the therapeutic relevant DOX concentration. Although we do not detect any differences in cell viability associated with BPA/DOX interaction long-term consequences on cell fate cannot be excluded. Taken together, the results presented here clearly reveal that it is essential to take into account the effects of interactions in BPA risk assessment, particularly in the context of chemotherapy.

5.7 References

Apostolou P, Toloudi M, Ioannou E, Chatziioannou M, Kourtidou E, Vlachou I, et al. 2013. Ap-1 gene expression levels may be correlated with changes in gene expression of some stemness factors in colon carcinomas. J Signal Transduct 2013:497383.

Belt EJ, Brosens RP, Delis-van Diemen PM, Bril H, Tijssen M, van Essen DF, et al. 2012. Cell cycle proteins predict recurrence in stage ii and iii colon cancer. Ann Surg Oncol 19 Suppl 3:S682-692.

Bolli A, Bulzomi P, Galluzzo P, Acconcia F, Marino M. 2010. Bisphenol a impairs estradiol-induced protective effects against dld-1 colon cancer cell growth. IUBMB Life 62:684-687.

Braniste V, Jouault A, Gaultier E, Polizzi A, Buisson-Brenac C, Leveque M, et al. 2010. Impact of oral bisphenol a at reference doses on intestinal barrier function and sex differences after perinatal exposure in rats. Proc Natl Acad Sci USA 107:448-453.

Center MM, Jemal A, Smith RA, Ward E. 2009. Worldwide variations in colorectal cancer. CA Cancer J Clin 59:366-378.

Durchdewald M, Angel P, Hess J. 2009. The transcription factor fos: A janus-type regulator in health and disease. Histol Histopathol 24:1451-1461.

Edginton AN, Ritter L. 2009. Predicting plasma concentrations of bisphenol a in children younger than 2 years of age after typical feeding schedules, using a physiologically based toxicokinetic model. Environ Health Perspect 117:645-652.

Edvardsson K, Nguyen-Vu T, Kalasekar SM, Ponten F, Gustafsson JA, Williams C. 2013. Estrogen receptor beta expression induces changes in the microrna pool in human colon cancer cells. Carcinogenesis 34:1431-1441.

EFSA European Food Safety Authority 2006. Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food on a request from the commition related to 2.2 - bis(4-hydroxyphenyl) propane (bisphenol A).The EFSA Journal 428: 1-75. Available at: "http://www.efsa.europa.eu/fr/efsajournal/pub/428.htm". [accessed February 2014].

Eom YW, Kim MA, Park SS, Goo MJ, Kwon HJ, Sohn S, et al. 2005. Two distinct modes of cell death induced by doxorubicin: Apoptosis and cell death through mitotic catastrophe accompanied by senescence-like phenotype. Oncogene 24:4765-4777.

EPA US. Environmental Protection Agency 1993. Integrated Risk Information System (IRIS) - Bisphenol A. Available at: "http://www.epa.gov/iris/subst/0356.htm". [accessed February 2014]

Fisher JW, Twaddle NC, Vanlandingham M, Doerge DR. 2011. Pharmacokinetic modeling: Prediction and evaluation of route dependent dosimetry of bisphenol a in monkeys with extrapolation to humans. Toxicol Appl Pharmacol 257:122-136.

Geens T, Aerts D, Berthot C, Bourguignon JP, Goeyens L, Lecomte P, et al. 2012. A review of dietary and non-dietary exposure to bisphenol-a. Food Chem Toxicol 50:3725-3740.

Ginsberg G, Rice DC. 2009. Does rapid metabolism ensure negligible risk from bisphenol a? Environ Health Perspect 117:1639-1643.

Greene RF, Collins JM, Jenkins JF, Speyer JL, Myers CE. 1983. Plasma pharmacokinetics of adriamycin and adriamycinol: Implications for the design of in vitro experiments and treatment protocols. Cancer Res 43:3417-3421.

Jin G, Howe PH. 1999. Transforming growth factor beta regulates clusterin gene expression via modulation of transcription factor c-fos. Eur J Biochem 263:534-542.

Kim HS, Lee YS, Kim DK. 2009. Doxorubicin exerts cytotoxic effects through cell cycle arrest and fas-mediated cell death. Pharmacology 84:300-309.

LaPensee EW, Tuttle TR, Fox SR, Ben-Jonathan N. 2009. Bisphenol a at low nanomolar doses confers chemoresistance in estrogen receptor-alpha-positive and -negative breast cancer cells. Environ Health Perspect 117:175-180.

LaPensee EW, LaPensee CR, Fox S, Schwemberger S, Afton S, Ben-Jonathan N. 2010. Bisphenol a and estradiol are equipotent in antagonizing cisplatin-induced cytotoxicity in breast cancer cells. Cancer Lett 290:167-173.

Mazzarelli P, Pucci S, Spagnoli LG. 2009. Clu and colon cancer. The dual face of clu: From normal to malignant phenotype. Adv Cancer Res 105:45-61.

Nahar MS, Soliman AS, Colacino JA, Calafat AM, Battige K, Hablas A, et al. 2012. Urinary bisphenol a concentrations in girls from rural and urban egypt: A pilot study. Environ Health 11:20.

Nikonova AS, Astsaturov I, Serebriiskii IG, Dunbrack RL, Jr., Golemis EA. 2013. Aurora a kinase (aurka) in normal and pathological cell division. Cell Mol Life Sci 70:661-687.

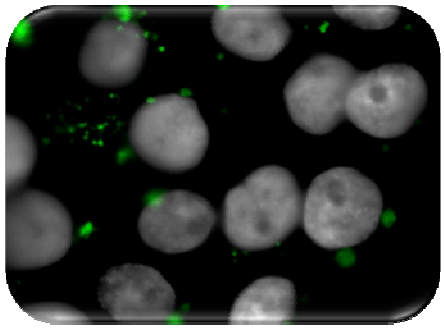
Park SS, Kim MA, Eom YW, Choi KS. 2007. Bcl-xl blocks high dose doxorubicin-induced apoptosis but not low dose doxorubicin-induced cell death through mitotic catastrophe. Biochem Biophys Res Commun 363:1044-1049.

Pennie WD, Aldridge TC, Brooks AN. 1998. Differential activation by xenoestrogens of er alpha and er beta when linked to different response elements. J Endocrinol 158:R11-14.

Poehlmann A, Reissig K, Just A, Walluscheck D, Hartig R, Schinlauer A, et al. 2013. Non-apoptotic function of caspases in a cellular model of hydrogen peroxide-associated colitis. J Cell Mol Med 17:901-913.

Ptak A, Wrobel A, Gregoraszczuk EL. 2011. Effect of bisphenol-a on the expression of selected genes involved in cell cycle and apoptosis in the ovcar-3 cell line. Toxicol Lett 202:30-35.
Pucci S, Mazzarelli P, Nucci C, Ricci F, Spagnoli LG. 2009. Clu "In and out": Looking for a link. Adv Cancer Res 105:93-113.
Ravizza R, Gariboldi MB, Passarelli L, Monti E. 2004. Role of the p53/p21 system in the response of human colon carcinoma cells to doxorubicin. BMC Cancer 4:92.
Ribeiro-Varandas E, Viegas W, Pereira HS, Delgado M. 2013. Bisphenol a at concentrations found in human serum induces aneugenic effects in endothelial cells. Mutat Res 751:27-33.
Rubin BS. 2011. Bisphenol a: An endocrine disruptor with widespread exposure and multiple effects. J Steroid Biochem Mol Biol 127:27-34.
Salameh A, Galvagni F, Anselmi F, De Clemente C, Orlandini M, Oliviero S. 2010. Growth factor stimulation induces cell survival by c-jun. Atf2-dependent activation of bcl-xl. J Biol Chem 285:23096-23104.
Soto AM, Sonnenschein C. 2010. Environmental causes of cancer: Endocrine disruptors as carcinogens. Nat Rev Endocrinol 6:363-370.
Tanaka M, Ueda A, Kanamori H, Ideguchi H, Yang J, Kitajima S, et al. 2002. Cell-cycle-dependent regulation of human aurora a transcription is mediated by periodic repression of e4tf1. J Biol Chem 277:10719-10726.
Taylor JA, Vom Saal FS, Welshons WV, Drury B, Rottinghaus G, Hunt PA, et al. 2011. Similarity of bisphenol a pharmacokinetics in rhesus monkeys and mice: Relevance for human exposure. Environ Health Perspect 119:422-430.
Tranoy-Opalinski I, Legigan T, Barat R, Clarhaut J, Thomas M, Renoux B, et al. 2014. Beta-glucuronidase-responsive prodrugs for selective cancer chemotherapy: An update. Eur J Med Chem 74:302-313.
Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgartten FJ, Schoenfelder G. 2010. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol a. Environ Health Perspect 118:1055-1070.
Venkatakrishnan CD, Dunsmore K, Wong H, Roy S, Sen CK, Wani A, et al. 2008. Hsp27 regulates p53 transcriptional activity in doxorubicin-treated fibroblasts and cardiac h9c2 cells: P21 upregulation and g2/m phase cell cycle arrest. Am J Physiol Heart Circ Physiol 294:H1736-1744.
Völkel W, Colnot T, Csanady GA, Filser JG, Dekant W. 2002. Metabolism and kinetics of bisphenol a in humans at low doses following oral administration. Chem Res Toxicol 15:1281-1287.
Völkel W, Bittner N, Dekant W. 2005. Quantitation of bisphenol a and bisphenol a glucuronide in biological samples by high performance liquid chromatography-tandem mass spectrometry. Drug Metab Dispos 33:1748-1757.
Warfel NA, El-Deiry WS. 2013. P21waf1 and tumourigenesis: 20 years after. Curr Opin Oncol 25:52-58.
WHO World Health Organization 2014. Cancer. Fact sheet n°297. Available at: "http://www.who.int/mediacentre/factsheets/fs297/en/". [accessed February 2014].

6 Cytotoxicity of *Eupatorium cannabinum L.* ethanolic extract against colon cancer cells and interactions with Bisphenol A and Doxorubicin



Edna Ribeiro-Varandas, Filipe Ressureição, Wanda Viegas, Margarida Delgado. Cytotoxicity of Eupatorium cannabinum L. extract against colon cancer cells and interactions with Bisphenol A and Doxorubicin. Accepted for publication in BMC Complementary and Alternative Medicine, manuscript ID: 2747237721217897, 2014.

6.1 Abstract

Background: *Eupatorium cannabinum L.* has long been utilized in traditional medicine, however no information is available regarding cellular effects of full extracts. The present work aimed to assess the effects of *E. cannabinum* ethanolic extract (EcEE) on the colon cancer line HT29 as well as to evaluate potential interactions with bisphenol A (BPA) a synthetic phenolic compound to which humans are generally exposed and doxorubicin (DOX) a commonly used chemotherapeutic agent.

Methods: HT29 cells were exposed to different concentrations (0.5 to 50 µg/ml) of EcEE alone or in combination with BPA or DOX. Cell viability was analyzed through resazurin assay. Gene transcription levels for *NCL, FOS, p21*, *AURKA* and *bcl-xl* were determined through qRT-PCR. Cytological analysis included evaluation of nuclear and mitotic anomalies after DAPI staining, immunodetection of histone H3 lysine 9 acetylation (H3K9ac) and assessment of DNA damage by TUNEL assay.

Results: Severe loss of HT29 cell viability was detected for 50 µg/ml EcEE immediately after 24 h of exposure whereas the lower concentrations assayed (0.5, 5 and 25 µg/ml) resulted in significant viability decreases only after 96 h. Exposure to 25 µg/ml EcEE for 48 h resulted in irreversible cell damage leading to a drastic decrease in cell viability after 72 h recovery in EcEE-free medium. The 48 h 25 µg/ml EcEE treatment also induced alteration of colony morphology, H3K9 hyperacetylation, transcriptional up regulation of *p21* and down regulation of *NCL, FOS* and *AURKA*, indicating reduced proliferation capacity. This treatment also resulted in drastic mitotic and nuclear disruption denoting induction of cell death. However, this was accompanied by up-regulation of *bcl-xl*, limited TUNEL labeling and nuclear size increase suggestive of a non-apoptocic cell death pathway. EcEE/BPA co-exposure increased mitotic anomalies particularly for the lowest EcEE concentration, although without major effects on viability. Conversely, EcEE/DOX co-exposure decreased cell viability in relation to DOX for all EcEE concentrations, without affecting the DOX-induced cell cycle arrest.

Conclusions: EcEE has effective cytotoxic activity on HT29 cancer cells leading to mitotic disruption and non-apoptotic cell death without severe induction of DNA damage. Interactions experiments showed that EcEE can increase BPA aneugenic effects. On the other hand, EcEE synergistic effects with DOX support a potential use as adjuvant in chemotherapeutic approaches.

6.2 Introduction

Eupatorium cannabinum L., commonly known as hemp-agrimony is a robust perennial herbaceous plant of the Asteraceae family, the only species of the Eupatorium genus found in Europe occurring also throughout North Africa and Asia (Schmidt and Schilling 2000). *E. cannabinum* has long been used for medicinal proposes being referred by Greeks and Romans as well by the medieval Persian physician Aviccena, for what is also known as Eupatorium of Aviccena, and later in by the Portuguese Renaissance pioneer in tropical medicine Garcia da Orta (1563). Presently, hemp-agrimnony is used in both Chinese (Fu et al. 2002) and Indian (Roeder and Wiedenfeld 2013) traditional medicines as well as in natural medicine in western countries (Kozel 1982) with very diverse therapeutic indications including influenza-like illnesses (Jaric et al. 2007), hypertension (Fu et al. 2002; Jaric et al. 2007; Roeder and Wiedenfeld 2013) and as an antitumoural agent (Roeder and Wiedenfeld 2013). *E. cannabinum* extracts has been previously characterized revealing the presence of sesquiterpenes (Rucker et al. 1997), pyrrolizidine alkaloids (Boppre et al. 2008; Fu et al. 2002) as well as several phenolic compounds (Chen et al. 2011; Zhang et al. 2008).

Sesquiterpenes were found to be a major fraction (43.3%) of essential oil from *E. cannabinum* aerial parts (Paolini et al. 2005), being eupatoriopicrin the main component (Rucker et al. 1997). Eupatoriopicrin has been associated with induction DNA damage in Ehrlich ascites tumour (Woerdenbag et al. 1989b) as well as with cytostatic activity and tumour growth inhibition proprieties in Lewis lung carcinoma and FIG26 fibrosarcoma both *in vitro* and *in vivo* (Woerdenbag et al. 1989a).

Pyrrolizidine alkaloids are generally associated with genotoxicity and tumourigenic activities (Fu et al. 2004), however the isomers intermedine and lycopsamine indentified in *E. cannabinum* have low genotoxic potency (Chen et al. 2010) and lycopsamine was shown to be non-tumourigenic in rats (Xia et al. 2013). Additionally the phenolic compounds identified in this plant have been described to have anti-inflammatory (Chen et al. 2011), anti-parasitary (Sulsen et al. 2007), as well as anti-proliferative effects in several cell lines (Forgo et al. 2012). In particular, jaceosidin cytotoxic effects have been demonstrated in normal and cancer endometrial cells (Lee et al. 2013) and hispidulin was shown to efficiently inhibit growth of gastric cancer cells (Yu et al. 2013) and liver carcinoma cells without significant toxic effect in normal liver cells (Gao et al. 2013).

Although the effects of specific components of *Eupatorium cannabinum L.* extracts have been described, the cellular effects of the full extracts have not, until now, been investigated. Thus, here we evaluated the effects of different concentrations of *Eupatorium cannabinum L.* ethanolic extract (EcEE) in the colon cancer cell line HT29. Moreover we also analyzed its interactions with the synthetic phenolic compound bisphenol A (BPA) as well as with the chemotherapeutic agent Doxorubicin (DOX). Human exposure to BPA is considered generalized in the common population and its adverse health effects are the focus of intense investigation (Vandenberg et al. 2009; Vandenberg et al. 2010). On the other hand, DOX is a common chemotherapeutic agent used in the treatment of a variety of cancers to which cell resistance can emerge (Doublier et al. 2008; Riganti et al. 2009). Plant constituents are a major source of bioactive compounds and several pants have been investigated aiming to identify potential synergistic effects with DOX (reviewed in Kapadia et al. 2013).

6.3 Materials and methods

Eupatorium cannabinum L. **ethanolic extract**

Eupatorium cannabinum L. (Asteraceae) aerial parts were collected in the Rossas fields of Arouca village, Portugal, in August during mass flowering. Formal identification of plant material was performed by A.P. Paes from “João

de Carvalho e Vasconcellos Herbarium" at Instituto Superior de Agronomia (Lisboa, Portugal). A voucher specimen was deposited in the same herbarium under the number LISI 1503/2013. Plant material was dried and powdered using a grinder and ethanolic extract (EcEE) was obtained by soaking the material in absolute ethanol for 48 h at room temperature with gentle shaking. The extract were filtered and concentrated under vacuum on a rotary evaporator at 40º C and stored at -20ºC for further use.

Cellular cultures, reagents and treatments

HT29 cells were purchased from European Collection of Cell Cultures (ECACC, UK) and cultivated in RPMI medium under standard conditions as previously described (Ribeiro-Varandas et al. 2013) Before treatments and experiments HT29 cells were allowed to stabilize for 24 h in standard medium and further cultivated in EcEE supplemented media for 24 h, 48 h or 96 h. Crude ethanolic extract was dissolved in ethanol to a final work concentration of 50 mg/ml before use and added to the culture media at four different final concentrations (0.5 µg/ml, 5 µg/ml, 25 µg/ml and 50 µg/ml). Bisphenol A (Sigma) was freshly diluted in ethanol and added to the culture media to the final concentration of 1 µg/ml (4.4 µM) that corresponds to the established Tolerable Daily Intake (TDI) level of 50 ug/kg BW/day (EFSA 2006, 2010) considering an average body weight of 70 Kg and daily consumption of 3 litres of preformed water. Doxorubicin (DOX) (AppliChem) was dissolved in water at stock concentration of 1 mg/ml and added to the culture media to final concentration of 2.5 µg/ml (4 µM) which corresponds to a therapeutic dosage (Greene et al. 1983). For the combined EcEE/BPA or EcEE/DOX exposures, cells were pre-exposed to EcEE for 24 h followed by additional 24 h of simultaneous exposure to EcEE and BPA or EcEE and DOX. Single 24 h BPA or DOX exposure was carried-out in equivalent cell cultures. For evaluation of cell recovery capacity after treatments cells were cultivated for additional 72 h in standard culture medium. Negative controls were performed for all experiments using cells grown in standard culture medium as well as cells grown in medium supplemented with ethanol at final concentration of 170 µM, corresponding to the final concentration of ethanol used as vehicle for all EcEE concentrations as well as for BPA.

Cell viability

Cell viability was evaluated by CellTiter-Blue assay (Promega) following manufacturer's instructions. Cells were plated on 96-well dishes at a density of 3.2×10^4 cells/well and after treatments were incubated for 4 h with CellTiter-Blue Reagent. Additional negative controls were performed in the absence of cells to guarantee that the utilized media did not interfere with fluorescence readings. Experiments were repeated at least three times with a minimum of three replicates per experiment.

DAPI staining, TUNEL assay and immunodetection

For cytological analysis cells were grown over glass coverslips coated with 0.2% (v/v) gelatin (Sigma) and after treatments fixed in 4% (p/v) formaldehyde in PBS. For evaluation of colony morphology, mitotic index as well as mitotic and nuclear anomalies cells were DAPI stained and mounted on glass slides with antifade AF1 (Citifluor). DNA damage assessment with TUNEL assay (Roche) was performed accordingly to manufacturers´ instructions. Immunodetection of H3K9ac and α-tubulin was performed in fixed cells as previously described (Ribeiro-Varandas et al. 2013) using the primary antidodies anti-acetyl-histone H3(Lys 9) (ab10812, Abcam) and anti-α-Tubulin (T9026, Sigma) detected with FITC or Cy3 conjugated secondary antibodies. Images were captured using the appropriate excitation and emission filters and recorded using an epifluorescence microscope Zeiss Axioskop2 equipped with a Zeiss AxioCam MRc5 digital camera. ImageJ software (http://rsbweb.nih.gov/ij/) was used for nuclear area measurements. The analysis was performed in the pooled results of at least two independent experiments with at least two replicates.

cDNA isolation and real-time quantitative PCR

Transcriptional analysis was performed by quantitative real-time PCR (qRT-PCR) for the proliferation-associated genes nucleolin (*NCL*), *FOS* and *p21*, for the cell cycle related gene *AURKA*, and the anti-apoptotic gene *bcl-xl*. The specific primers utilized are listed in Table 1, *GAPDH* and *β-actin* were used as control genes (Ribeiro-Varandas et al. 2013; 2014). Total RNA was extracted

from trypsinized cells with the RNAqueous Kit (Invitrogen) following manufacturers´ instructions. 3 µg of total RNA was utilized for RNase free DNase digestion (RQ1 RNase free DNase, Promega) and first strand cDNA synthesis was completed with random primers (DYNAmo cDNA syntesis Kit, Thermo Scientific). The resulting cDNA was utilized for qRT-PCR with SsoFast Eva Green Supermix (BioRad) utilizing the following conditions: 95 ◦C for 3 min, 35 cycles (95 ºC for 30 s, 55 ºC for 30 s, 72 ºC for 40 s), and 72 ◦C for 5 min. Control PCRs were also performed prior to cDNA synthesis and for all primer combinations without template. Experiments were repeated at least three times with at least three replicates per cell treatment/primer combination in each experiment. Since no significant differences were detected between the two reference genes, threshold cycles (Ct) of the target genes were equilibrated with the mean Ct of GAPDH and β-actin genes to calculate ΔCt ($\Delta Ct = Ct_{target} - \text{mean } Ct_{GAPDH:\beta\text{-actin}}$). Gene expression levels were analyzed by calculating ΔΔCt ($\Delta\Delta Ct = \Delta Ct_{treatment} - \text{mean } \Delta Ct_{control}$). Results are presented as log2 of the mean fold change ($2^{-\Delta\Delta Ct}$) ± standard deviation.

Table 1 - Primers used for qRT-PCR

Gene	Accession na	Forward primer (5´→3´)	Reverse primer (5´→3´)
p21	NM_000389	CTGGAGACTCTCAGGGTCGAA	CCAGGACTGCAGGCTTCCT
AURKA	NM_003600	GCTGGAGAGCTTAAAATTGCAG	TTTTGTAGGTCTCTTGGTATGTG
FOS	NM_005252	AGGAGAATCCGAAGGGAAAG	CAAGGGAAGCCACAGACATC
Bcl-xl	NM_001191.2	TTACCTGAATGACCACCTA	ATTTCCGACTGAAGAGTGA
NCL	NM_005381	CCTTCTGAGGACATTCCAAGACA	ACGGTATTGCCCTTGAAATGTT
GAPDH	NM_002046	GAGTCAACGGATTTGGTCGTA	GCAGAGATGATGACCCTTTTG
β-actin	NM_001101	GGTCATCTTCTCGCGGTTGGCCTTGGGGT	CCCCAGGCACCAGGGCGTGAT

[a] GenBank accession numbers (National Center for Biotechnology).

Statistical analysis

Student´s t test was use for statistic analysis of gene transcription, cell viability, nuclear area and nuclear fragmentation. No significant differences were detected between control and vehicle for all parameters analysed, results are shown in relation to control. GraphPad Prism 6 software was used for determination of IC_{50} values.

6.4 Results

6.4.1 *E. cannabinum* ethanolic extract decreases HT29 cell viability.

Assessment of cell viability was performed to test potential cytotoxic effects of *E. cannabinum* ethanolic extract (EcEE) on HT29 cells. For this, CellTiter-Blue assay was utilized and effects of different concentrations of EcEE (0.5 µg/ml, 5 µg/ml, 25 µg/ml and 50 µg/ml) were evaluated after 24 h, 48 h and 96 h of exposure (Figure 1-A). The higher EcEE concentration (50 µg/ml) resulted in severe cell viability decrease after 24 h exposure and complete loss of viability at subsequent time points analyzed (48 h and 96 h). On the other hand, no decrease in cell viability was detected after 24 h or 48 h for the lower EcEE concentrations, and a slight increase in fluorescence was observed after 24 h and 48 h for 25 µg/ml EcEE. However, after 96 h of exposure significant decreases in cell viability were detected for the three lower EcEE concentrations, and particularly for 25 µg/ml EcEE (-18.89%, -14.55% and -73.25% for 0.5 µg/ml, 5 µg/ml and 25 µg/ml, respectively). Taken together, these results indicate more severe effects after prolonged exposure. This is further shown by IC_{50} values of 46.75, 44.64 and 13.38 µg/ml for 24, 48 and 96 h, respectively. To detect possible deferred effects of the EcEE exposure cell viability was also evaluated after 72 h of recovery in standard culture media (Figure 1-B) revealing a severe decrease exclusively for 48 h exposure to 25 µg/ml EcEE (-75.59%). To better understand the effects of EcEE immediately after 48 h exposure, gene transcription analysis was carried out for three proliferation-associated genes, namely nucleolin (*NCL)*, *p21* and *FOS* (Figure 1-C). Similarly to the cell viability results, no significant differences in transcription levels were detected after 48 h exposure to EcEE concentrations equal or lower to 5 µg/ml. Conversely, 25 µg/ml EcEE exposure resulted in significant differences in mRNA levels of all three genes, corresponding to down regulation of both *NCL* and *FOS* (Log2 fold change = -0.813 ± 0.248 and -0.741 ± 0.078, respectively), and up regulation of *p21* (Log2 fold change = 1.393 ± 0.128). Evaluation of colony morphology was performed immediately after EcEE treatments by DAPI staining. Again, significant alterations in colony morphology were detected after exposure to 25 µg/ml EcEE for 48 h, evident as cells being more dispersed showing a flattening of cellular aggregates in comparison to

controls with no detectable effect for 5 µg/ml EcEE (Figure 1-D) or 0.5 µg/ml EcEE (not shown).

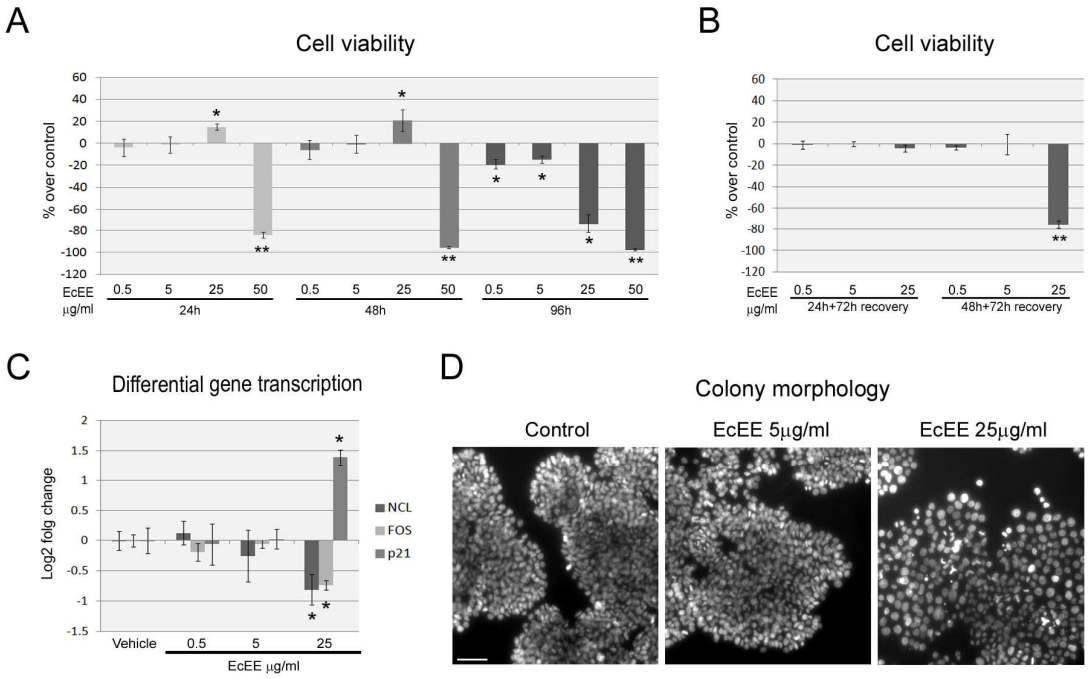


Figure 1 - EcEE affect cell viability and proliferation (A) Cell viability after 24 h, 48 h and 96 h of exposure to distinct concentrations of EcEE and (B) after 72 h recovery in EcEE-free medium following 24 h and 48 h treatments. Results are presented as percentage over control, **p<0.0001 and *p<0.01. (C) NCL, FOS and p21 differential transcription after 48 h exposure to distinct EcEE concentrations. Results are shown as mean log2 fold change ($2^{-\Delta\Delta Ct}$) ± standard deviation in relation control, *p<0.0001. (D) DAPI stained HT29 colonies after 48 h in control medium and medium supplemented with EcEE 5 µg/ml or EcEE 25 µg/ml. All images have identical magnification, bar = 50 µm.

6.4.2 *E. cannabinum* ethanolic extract induces alterations in nuclear structure and mitotic disruption.

A detailed cytological analysis was performed for 0.5 µg/ml, 5 µg/ml and 25 µg/ml EcEE concentrations after 48 h of exposure and again significant nuclear alterations were observed exclusively for 25 µg/ml EcEE (Figure 2-A). This was obvious as the prominent occurrence of micronuclei and highly condensed nuclei (pyknosis) scattered throughout cell aggregates as well as fragmented nuclei (karyorrhexis), revealing irreversible nuclear damage. In addition, TUNEL assay showed that induction of DNA breaks also occurred after 48 h exposure

to 25 µg/ml EcEE treatments although at a much lower level than nuclear abnormalities, as many of the abnormal nuclei were not TUNEL positive and in positive nuclei labeling was sparse (Figure 2-B). For the two lower EcEE concentrations (0.5 and 5 µg/ml) no TUNEL positive nuclei were detected (not shown) as observed for control. Importantly, qRT-PCR transcriptional analysis of the anti-apoptotic *bcl-xl* gene revealed that EcEE exposure induced a significant up regulation of this gene not only at 25 µg/ml (Log2 fold change = 0.528 ± 0.243) but also at 5 µg/ml, although to a lesser extent (Log2 fold change = 0.158 ± 0.067) (Figure 2-C). Quantification of the nuclear area of non-pyknotic and non-fragmented DAPI stained nuclei showed a significant increase in this parameter in relation to control for cells exposed to 25 µg/ml EcEE but not to the lower EcEE concentrations (not shown). The increment in nuclear area after the 48 h exposure to 25 µg/ml EcEE corresponded in average to 48.8% ($n > 70$ for each growth condition, $p < 0.0001$ for 25 µg/ml EcEE in relation to control) and was accompanied by an evident increase in cellular area revealed by α-tubulin immunodetection (Figure 2-D). Moreover evident chromatin enrichment in histone H3 acetylated on lysine 9 (H3K9ac) was detected also for 48 h 25 µg/ml EcEE (Figure 1-E) whereas no alteration was observed for either 0.5 µg/ml or 5 µg/ml EcEE (not shown). The effects of exposure to EcEE were further evaluated on mitotic cells after DAPI staining. No significant variation was observed in the mitotic index between control, vehicle and EcEE independently of the concentration assayed (varying between 4.57 and 5.94). On the other hand, although mitotic anomalies, particularly multipolar metaphases and anaphases, are a common feature of HT29 cells and therefore observed both in control and vehicle (6.67% and 11.76% after 24 h; 6.38% and 10.67% after 48 h, for control and vehicle respectively), the percentage of abnormal mitosis increased after exposure to all EcEE concentrations (Figures 3-A and 3-B). Although a slight increase of abnormal mitosis was already detectable for 0.5 µg/ml EcEE, this effect was greater for 5 µg/ml EcEE (41% and 44% after 24 h or 48 h, respectively). After exposure to 25 µg/ml EcEE, most mitotic cells presented abnormalities (80% and 63% after 24 and 48 h, respectively). Although the frequency of abnormal mitosis was greater after 24 h at the higher EcEE concentration, these results clearly

indicate that EcEE induces mitotic disruption in a dose dependent manner. Interestingly, qRT-PCR analysis revealed a significant down regulation of *AURKA* (Log2 fold change = -0.938 ± 0.146), a gene that encodes a key protein for mitotic chromosome segregation.

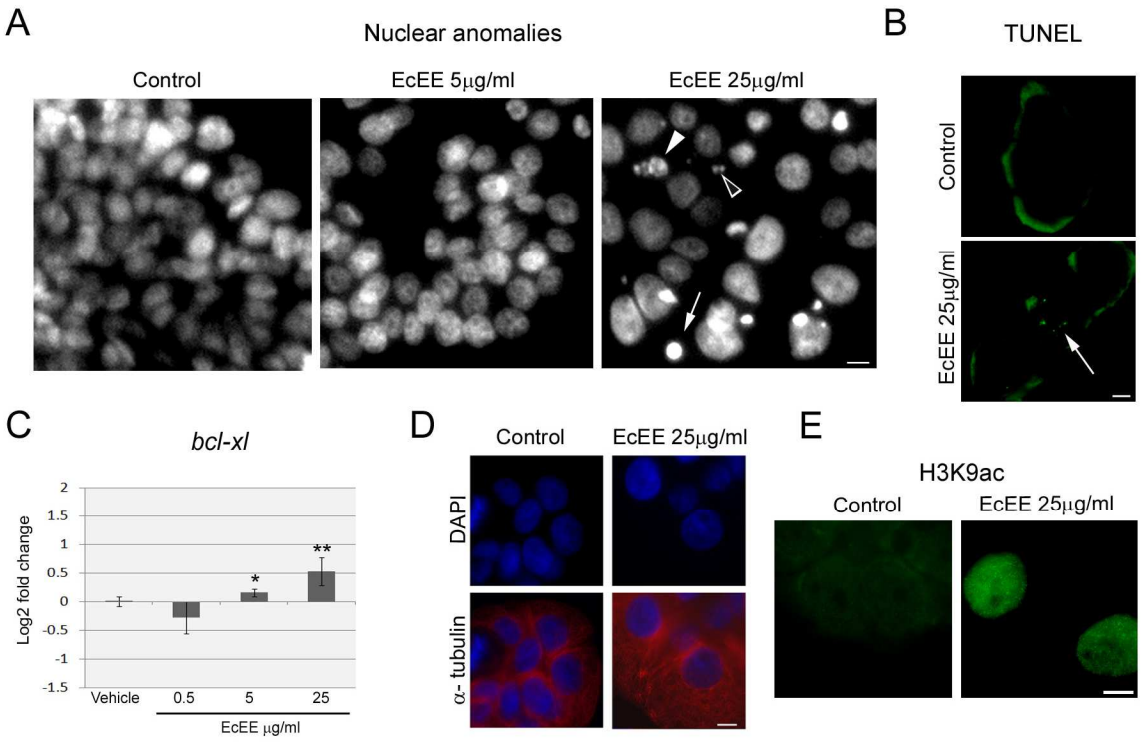


Figure 2 - Nuclear organization is disrupted after 48 h of exposure to EcEE 25 µg/ml (A) DAPI stained HT29 interphase cells. Nuclear anomalies, namely pyknosis (arrow), micronuclei (open arrow head) and karyorrhexis (arrow head) are detectable only for 25 µg/ml EcEE. The lack of effects induced by EcEE lower concentrations is exemplified by 5 µg/ml EcEE. (B) TUNEL positive nuclei (arrow) with sparse labeling are detectable for 25 µg/ml EcEE. (C) bcl-xl differential expression after 48 h exposure to distinct EcEE concentrations. Results in are shown as the mean log2 fold change ($2^{-\Delta\Delta Ct}$) ± standard deviation in relation control, **p<0.0001 and *p<0.01. (D) DAPI staining (blue) and α-tubulin immunodetection (red in merged images at the bottom) of interphase cells after 48 h in control or 25 µg/ml EcEE. Merged images are shown in the bottom. (E) Immunodetection of H3K9ac after 48 h in control or EcEE 25 µg/ml supplemented medium. Within each experiment all images have identical magnification, bar = 5 µm.

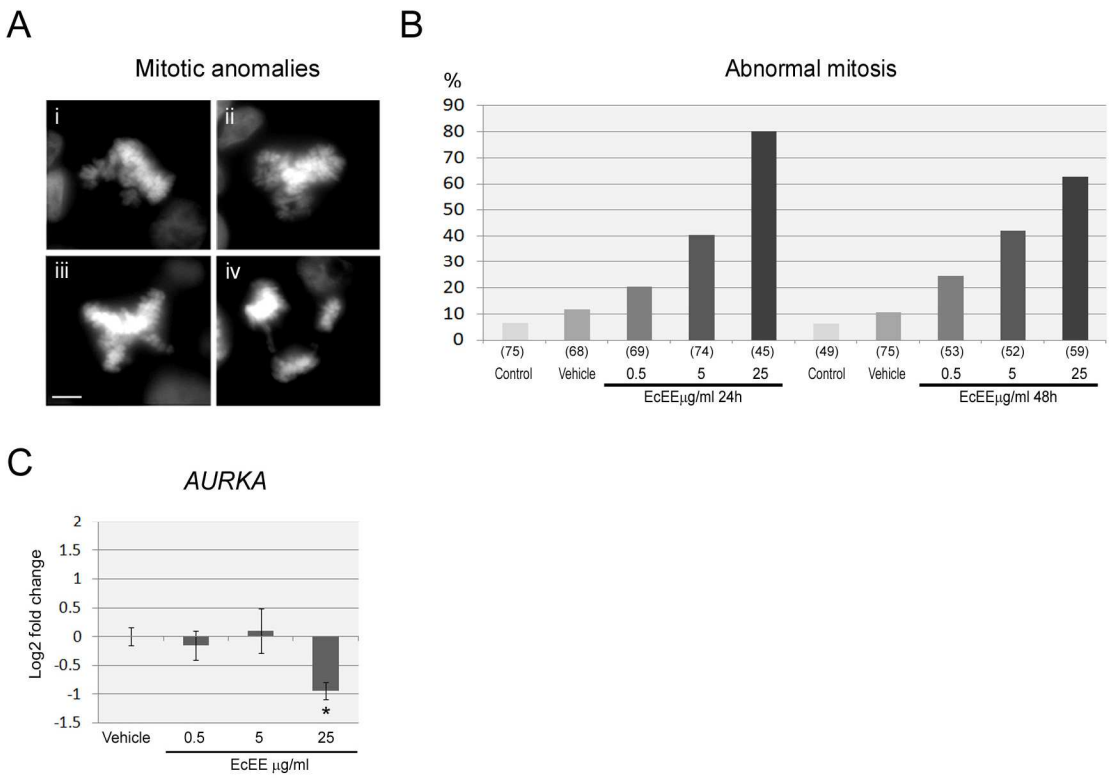


Figure 3 - EcEE exposure induces mitotic disruption. (A) DAPI stained abnormal mitotic cells after EcEE exposure showing (i) defective chromosome congression, (ii) tripolar and (iii) tetrapolar metaphases and (iv) tripolar anaphase with chromosome bridges, bar = 5μm. (B) Percentage of abnormal mitosis after 24 h and 48 h exposure to distinct concentrations of EcEE; the total number of mitotic cells scored to calculate the percentage of abnormal mitosis is shown in brackets. (C) AURKA differential transcription after exposure to 48 h of EcEE. Results are shown as the mean log2 fold change ($2^{-\Delta\Delta Ct}$) ± standard deviation in relation control, *p<0.0001.

6.4.3 *E. cannabinum* ethanolic increases Bisphenol A induced mitotic disruption.

Interactions between EcEE and the environmental pollutant BPA at reference level (1 μg/ml) were evaluated. Co-exposure to EcEE and BPA did not affect cell viability immediately after treatments, as no significant differences were detected in relation to control (Figure 4-A). After 72 h recovery in standard medium a severe decrease in cell viability (-93.48%) was exclusively observed for 25 μg/ml EcEE/BPA (Figure 4-A) being even stronger than that observed for 25 μg/ml EcEE alone (Figure 1-B).

Cytological evaluation of mitotic disruption after DNA DAPI staining (Figure 4-B) revealed that BPA exposure alone increased the level of mitotic anomalies to

20.5%. Interestingly, a stronger effect of BPA co-exposure was observed for the lowest EcEE concentration assayed (0.5 µg/ml) resulting in 41% of abnormal mitosis (Figure 4-B) compared to 25% observed for EcEE alone (Figure 3-B). In contrast, no evident effect of BPA was detected for the intermediate EcEE concentration as an identical level of 44% was detected for 5 µg/ml EcEE alone or in combination with BPA. Co–exposure to the higher EcEE concentration (25 µg/ml) and BPA resulted in a particular high level of mitotic anomalies (75%), although the difference in relation to EcEE alone (63%) was smaller than that observed for the lower EcEE concentration.

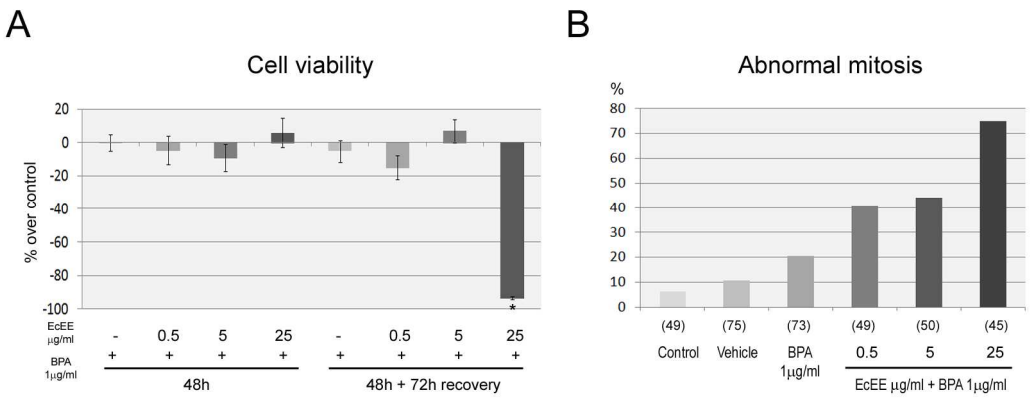


Figure 4 - EcEE interacts with BPA at reference level (A) Cell viability after co-exposure to BPA (1 µg/ml) and distinct EcEE concentrations and subsequent 72 h recovery in standard medium. Results are presented as percentage over control, *p<0.001. (B) Percentage of mitotic anomalies after 48 h culture in standard medium (control) or medium supplemented with ethanol (vehicle), BPA or EcEE at distinct concentrations in combination with BPA. The total number of mitotic cells scored to calculate the percentage of abnormal mitosis is shown in brackets.

6.4.4 Cytotoxic effects of Doxorubicin are enhanced by *E. cannabinum.*

Potential interactions between different concentrations of EcEE and the chemotherapeutic drug doxorubicin (DOX) at a therapeutic concentration of 2.5 µg/ml were investigated. Immediately after exposure, DOX alone resulted in a slight decrease in cell viability (-4.15%) (Figure 5-A). Interestingly, the loss of cell viability was significantly more pronounced after co-exposure to EcEE/DOX for all EcEE concentrations (-10.03%, -19.88% and -18.67% for 0.5 µg/ml, 5 µg/ml and 25 µg/ml, respectively) (Figure 5-A) contrasting with the lack of

effects observed for 48 h exposure to EcEE alone (Figure 1-A). Recovery experiments showed that the effects of both DOX and 25 µg/ml EcEE/DOX have long lasting negative effects on viability, apparent as prominent decreases in cell viability after 72 h recovery in standard medium in relation to what was observed immediately after exposure (Figure 5-A). After recovery, EcEE 25 µg/ml/DOX exposure resulted in an even more pronounced loss of cell viability (-93.95%) than that observed for exposure to 25 µg/ml EcEE alone (Figure 1-B). Conversely, for the lower EcEE concentrations, no significant differences were detected between exposure to DOX alone and in combination with EcEE (Figure 1-B).

Cytological analysis after DAPI staining showed a complete absence of cells at mitosis after exposure to DOX alone or in combination to EcEE, independently of the EcEE concentration. Conversely both pyknotic cells and fragmented nuclei were observed after exposure to DOX alone or in combination to EcEE (Figure 5-B). Since identical nuclear alterations were also observed after single exposure to 25 µg/ml EcEE (Figure 2-E), the levels of nuclear fragmentation were compared between single exposure to 25 µg/ml EcEE or DOX alone and the combination of both (Figure 5-C). The results obtained revealed that the induction of nuclear fragmentation is significantly higher for 25 µg/ml EcEE/DOX combined exposure (20.28%) than for either DOX (7.63%) or 25 µg/ml EcEE (8.89%) alone.

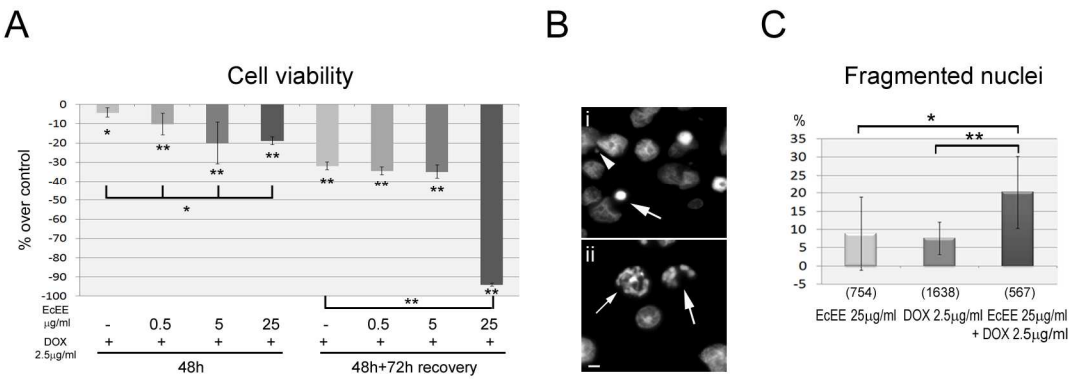


Figure 5 - EcEE has synergistic effects with DOX at a therapeutic dose. (A) Cell viability after co-exposure to DOX (2.5 µg/ml) and distinct EcEE concentrations and subsequent 72 h recovery in standard medium. Results are presented as percentage over control, the level of significance in relation to DOX alone is indicated by horizontal brackets, **p<0.0001 and *p<0.01. (B) DAPI stained cell after co-exposure to EcEE 25 µg/ml/DOX illustrating the occurrence of (i) micronuclei (arrow head) and pyknotic nuclei (arrow) and (ii) fragmented nuclei (arrows), bar = 5 µm. (C) Percentage fragmented nuclei after exposure to EcEE 25 µg/ml or DOX alone and the combination of both. Total number of cells analyzed is shown in brackets, **p<0.0001 and *p<0.03 in relation to EcEE 25 µg/ml or DOX alone.

6.5 Discussion

Eupatorium cannabinum L. is a commonly utilized plant for alternative and/or complementary medicine treatments (Jaric et al. 2007) including as an anticancer agent (Roeder and Wiedenfeld 2013). Although cellular effects of particular phytochemicals known to be present in *E. cannabinum* have been previously described, to our knowledge this is the first study that evaluates the cytotoxic potential of *E. cannabinum* extracts on human cancer cells. Here we demonstrated that *E. cannabinum* ethanolic extract (EcEE) has cytotoxic effects on HT29 colon cancer cells in a time and dose dependent manner. IC_{50} were similar after 24 and 48 h (46.75 and 44.65 µg/ml, respectively) but considerably lower (13.38 µg/ml) after 96 h of exposure. Cytotoxic activity has also been demonstrated for extracts from other *Eupatorium* species. For *E. perfoliatum* ethanolic extract, IC_{50} values between 12 and 14 µg/ml were obtained after 24h exposure in three distinct mammalian cell lines (Habtemariam and Macpherson 2000). In MCF7 breast cancer cells a time dependent effect was also observed for *E. odoratum* ethyl acetate extract (IC_{50} of 65.72, 83.88 µg/ml and 92.84

µg/ml for 24, 48 and 72 h, respectively) while for acetone extract higher IC_{50} values were obtained but without a direct correlation with exposure time (133.9, 163.0 and 147.8 µg/ml for 24, 48, and 72 h respectively) (Harun et al. 2012). The immediate cytotoxicity here observed for EcEE is lower than that observed for *E. perfoliatum* ethanolic extract but higher than that observed for ethyl acetate and acetone extracts from *E. odoratum*. Interestingly the time dependent increase in cytotoxicity of EcEE was only detected for the longer exposure time (96 h). Moreover a deferred effect on cell viability was detected after 48 h exposure to EcEE at 25 µg/ml. This was also associated with disruption of cell colony three-dimensional arrangement was associated with a generalized increase in nuclear area and H3K9 hyperacetylation. Relevantly, gene transcription analysis revealed a significant reduction in the mRNA levels of *FOS*, which encodes for a nuclear protein from AP-1 transcription factor complex, and nucleolin (*NCL*) the most profuse non-ribosomal protein of the nucleolus. Both *FOS* and nucleolin are involved in the regulation of cell proliferation (Mongelard and Bouvet 2007; Shaulian and Karin 2002) as their decreased expression has been related with reduced proliferation capacity of cancer cells including colon cancer cell lines (Pandey et al. 2012; Turck et al. 2004). On the other hand, exposure to EcEE (25 µg/ml, 48 h) also resulted in the up regulation of *p21*, a cyclin-dependent kinase inhibitor which is a major regulator of the cell cycle (Xiong et al. 1993). It was previously shown that histone hyperacetylation induces *p21* over expression (Fang and Lu 2002). In colon cancer cells inhibition of histone deacetylation results in both up regulation of *p21* (Druesne et al. 2004), and induction of G2/M cell cycle arrest (Robert et al. 2001). Relevantly, cell reduction capacity depends on the cell cycle being higher at G2/M (Conour et al. 2004). Considering that the cell viability assay used is based on the resazurin reduction and that overall our results were incompatible with EcEE induction of cell proliferation, the slight and transient augment of fluorescence detected after 24 h and 48 h of exposure to 25 µg/ml EcEE was also suggestive of cell arrest at G2 or M. Moreover, the increase of abnormal mitotic cells after exposure to EcEE is also suggestive of a mitotic block. This phenotype was accompanied by a significant down regulation of Aurora A transcription, what is consistent with previous results

showing that decreased Aurora A levels are associated with mitotic catastrophe and consequent cell death (Kimura et al. 2013). Induction of cell death after 48 h exposure to 25 µg/ml was evident by the prominent occurrence of pyknotic and fragmented nuclei, characteristic of both apoptotic as well as necrotic cells, and supports the marked loss in cell viability observed after recovery. This was moreover associated with transcriptional up regulation of the anti-apoptotic gene *bcl-xL* suggesting a non-apoptotic cell death pathway (Michels et al. 2013) also supported by limited occurrence of DNA breaks. These observations together with the increase in the cell size is compatible with a necrotic cell death or necroptosis, a process which acts as backup death-inducing mechanism when apoptosis is inhibited (Cerella et al. 2014).

Cytostatic activity was previously described for compounds identified in *E. cannabinum* extracts namely the sesquiterpene eupatoriopicrin (Rucker et al. 1997) and the flavonoids centaureidin, jaceosidin and hispidulin (Zhang et al. 2008). Severe decrease of tumour cell survival *in vitro* was associated with eupatoriopicrin concentrations ranging from 1-10 µg/ml (Woerdenbag et al. 1989a; Woerdenbag et al. 1989b) which was correlated with induction of DNA damage (Woerdenbag et al. 1989b). Also anti-proliferative effects on distinct cancer cell lines have been described for centaureidin concentrations below 1 µg/ml (Forgo et al. 2012) as well as for jaceosidin in the concentration range of 20-50 µg/ml (Lee et al. 2013) and hispidulin for 4-30 µg/ml (Gao et al. 2013). Relevantly, both jaceosidin (Lee et al. 2013) and hispidulin (Yu et al. 2013) effects were associated with increased *p21* expression. The results now obtained indicate that the anti-proliferative potency of EcEE is similar to some of its individual constituents in particular eupatoriopicrin, jaceosidin and hispidulin without marked induction of DNA damage suggesting a combined action of distinct compounds.

Importantly, EcEE combined exposures with DOX at therapeutic concentration resulted in a clear enhancement of cytotoxic effects evident as combined treatments significantly decreased HT29 cell viability immediately after exposure, also observed for the lower EcEE concentration that *per se* did not affect cell viability. This was accompanied by increased nuclear fragmentation and reduced cell survival after recovery resulting in almost total loss of cell

viability. DOX is a commonly utilized antineoplastic drug that acts in tumour cells by induction of apoptosis (Gamen et al. 2000). Nevertheless different types of cell death can occur simultaneously, independently or through partially common pathways (reviewed in Cerella et al. 2014). The severe decrease in cell viability observed after combined exposure to DOX and EcEE can thus result from induction of distinct cell death mechanisms. On the other hand therapeutic concentrations of DOX induces cell arrest at G2/M and/or G1/S checkpoints (Bar-On et al. 2007; Lupertz et al. 2010). The results obtained show that EcEE does not counteract DOX-induced cell cycle arrest. Considering that DOX acts by induction of apoptosis (Gamen et al. 2000) and to which cell resistance can emerge (Doublier et al. 2008; Riganti et al. 2009) our data substantiates potential adjuvant EcEE properties in chemotherapeutic approaches (Koehler et al. 2014).

On the other hand, no immediate effect on cell viability was associated with co-exposure to EcEE and the synthetic phenolic compound BPA. However, cell recovery capacity after 48 h exposure to 25 µg/ml EcEE was decreased by the presence of BPA. Additionally, EcEE/BPA combined exposures resulted in increased mitotic anomalies in relation to either BPA or EcEE alone for 25 µg/ml EcEE but also for 0.5 µg/ml EcEE. BPA is characterized as an aneugenic chemical (Johnson and Parry 2008) capable to interfere with cell division mechanisms even at very low concentrations (Ribeiro-Varandas et al. 2013). Nonetheless BPA is widely used in a variety of consumer products leading to a generalized human exposure and its risks remain highly controversial (Vandenberg et al. 2010). The present results raise the possibility that adverse BPA effects could be enhanced by interactions with other chemicals, an aspect that remains largely unknown and has barely been addressed.

6.6 Conclusion

E. cannabinum has been utilized as a medicinal plant for alternative and/or complementary medicine, however the effects or the mode of action of full extracts have not been evaluated at the cellular level. The present work demonstrates that *E. cannabinum* ethanolic extract has potent cytotoxic activity against HT29 colon cancer cells associated with mitotic disruption and cell

death without marked evidences of DNA damage. Relevantly *E. cannabinum* extract exhibits synergistic effects with doxorubicin in the induction of HT29 cell death indicating its potential use in alternative or complementary therapeutic strategies. On the other hand, the results show also that *E. cannabinum* can increase aneugenic effects of the environmental pollutant BPA, drawing attention to the possibility that BPA adverse effects may be potentiated by interaction with other chemicals.

6.7 References

Bar-On O, Shapira M, Hershko DD. 2007. Differential effects of doxorubicin treatment on cell cycle arrest and skp2 expression in breast cancer cells. Anticancer Drugs 18:1113-1121.

Boppre M, Colegate SM, Edgar JA, Fischer OW. 2008. Hepatotoxic pyrrolizidine alkaloids in pollen and drying-related implications for commercial processing of bee pollen. J Agric Food Chem 56:5662-5672.

Cerella C, Teiten MH, Radogna F, Dicato M, Diederich M. 2014. From nature to bedside: Pro-survival and cell death mechanisms as therapeutic targets in cancer treatment. Biotechnol Adv.

Chen JJ, Tsai YC, Hwang TL, Wang TC. 2011. Thymol, benzofuranoid, and phenylpropanoid derivatives: Anti-inflammatory constituents from eupatorium cannabinum. J Nat Prod 74:1021-1027.

Chen T, Mei N, Fu PP. 2010. Genotoxicity of pyrrolizidine alkaloids. J Appl Toxicol 30:183-196.

Conour JE, Graham WV, Gaskins HR. 2004. A combined in vitro/bioinformatic investigation of redox regulatory mechanisms governing cell cycle progression. Physiol Genomics 18:196-205.

Doublier S, Riganti C, Voena C, Costamagna C, Aldieri E, Pescarmona G, et al. 2008. Rhoa silencing reverts the resistance to doxorubicin in human colon cancer cells. Mol Cancer Res 6:1607-1620.

Druesne N, Pagniez A, Mayeur C, Thomas M, Cherbuy C, Duee PH, et al. 2004. Diallyl disulfide (dads) increases histone acetylation and p21(waf1/cip1) expression in human colon tumor cell lines. Carcinogenesis 25:1227-1236.

EFSA European Food Safety Authority 2006. Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a request from the Commition related to 2.2 - BIS(4-HYDROXYPHENYL) PROPANE (bisphenol A). EFSA Journal 2006, 428:1-75.

EFSA European Food Safety Authority 2010. Scientific opinion on Bisphenol A: evaluation on a study investigating its neurodevelopmental toxicity, review of recent scientific literature on its toxicity and advice on the Danish risk assessment of Bisphenol A. EFSA Journal 2010, 8:1829.

Fang JY, Lu YY. 2002. Effects of histone acetylation and DNA methylation on p21(waf1) regulation. World J Gastroenterol 8:400-405.

Forgo P, Zupko I, Molnar J, Vasas A, Dombi G, Hohmann J. 2012. Bioactivity-guided isolation of antiproliferative compounds from centaurea jacea l. Fitoterapia 83:921-925.

Fu PP, Yang Y, Xia Q., Chou MW, Cui YY, Lin G. 2002. Pyrrolizidine alkaloids - tumorigenic components in chinese herbal medicines and dietary supplements Journal of Food and Drug Analysis 10:198-211

Fu PP, Xia Q, Lin G, Chou MW. 2004. Pyrrolizidine alkaloids--genotoxicity, metabolism enzymes, metabolic activation, and mechanisms. Drug Metab Rev 36:1-55.
Gamen S, Anel A, Perez-Galan P, Lasierra P, Johnson D, Pineiro A, et al. 2000. Doxorubicin treatment activates a z-vad-sensitive caspase, which causes deltapsim loss, caspase-9 activity, and apoptosis in jurkat cells. Exp Cell Res 258:223-235.
Gao H, Wang H, Peng J. 2013. Hispidulin induces apoptosis through mitochondrial dysfunction and inhibition of p13k/akt signalling pathway in hepg2 cancer cells. Cell Biochem Biophys.
Greene RF, Collins JM, Jenkins JF, Speyer JL, Myers CE. 1983. Plasma pharmacokinetics of adriamycin and adriamycinol: Implications for the design of in vitro experiments and treatment protocols. Cancer Res 43:3417-3421.
Habtemariam S, Macpherson AM. 2000. Cytotoxicity and antibacterial activity of ethanol extract from leaves of a herbal drug, boneset (eupatorium perfoliatum). Phytother Res 14:575-577.
Harun FB, Syed Sahil Jamalullail SM, Yin KB, Othman Z, Tilwari A, Balaram P. 2012. Autophagic cell death is induced by acetone and ethyl acetate extracts from eupatorium odoratum in vitro: Effects on mcf-7 and vero cell lines. ScientificWorldJournal 2012:439479.
Jaric S, Popovic Z, Macukanovic-Jocic M, Djurdjevic L, Mijatovic M, Karadzic B, et al. 2007. An ethnobotanical study on the usage of wild medicinal herbs from kopaonik mountain (central serbia). J Ethnopharmacol 111:160-175.
Johnson GE, Parry EM. 2008. Mechanistic investigations of low dose exposures to the genotoxic compounds bisphenol-a and rotenone. Mutat Res 651:56-63.
Kapadia GJ, Rao GS, Ramachandran C, Iida A, Suzuki N, Tokuda H. 2013. Synergistic cytotoxicity of red beetroot (beta vulgaris l.) extract with doxorubicin in human pancreatic, breast and prostate cancer cell lines. J Complement Integr Med 10.
Kimura M, Yoshioka T, Saio M, Banno Y, Nagaoka H, Okano Y. 2013. Mitotic catastrophe and cell death induced by depletion of centrosomal proteins. Cell Death Dis 4:e603.
Koehler BC, Jager D, Schulze-Bergkamen H. 2014. Targeting cell death signaling in colorectal cancer: Current strategies and future perspectives. World J Gastroenterol 20:1923-1934.
Kozel C. 1982. Guía de medicina natural isbn:8485868005. Barcelona, spain.
Lee JG, Kim JH, Ahn JH, Lee KT, Baek NI, Choi JH. 2013. Jaceosidin, isolated from dietary mugwort (artemisia princeps), induces g2/m cell cycle arrest by inactivating cdc25c-cdc2 via atm-chk1/2 activation. Food Chem Toxicol 55:214-221.
Lupertz R, Watjen W, Kahl R, Chovolou Y. 2010. Dose- and time-dependent effects of doxorubicin on cytotoxicity, cell cycle and apoptotic cell death in human colon cancer cells. Toxicology 271:115-121.
Michels J, Kepp O, Senovilla L, Lissa D, Castedo M, Kroemer G, et al. 2013. Functions of bcl-x l at the interface between cell death and metabolism. Int J Cell Biol 2013:705294.
Mongelard F, Bouvet P. 2007. Nucleolin: A multifaceted protein. Trends Cell Biol 17:80-86.
Pandey MK, Liu G, Cooper TK, Mulder KM. 2012. Knockdown of c-fos suppresses the growth of human colon carcinoma cells in athymic mice. Int J Cancer 130:213-222.
Paolini J, Costa J, Bernardini AF. 2005. Analysis of the essential oil from aerial parts of eupatorium cannabinum subsp. Corsicum (l.) by gas chromatography with electron impact and chemical ionization mass spectrometry. J Chromatogr A 1076:170-178.
Ribeiro-Varandas E, Viegas W, Pereira HS, Delgado M. 2013. Bisphenol A at concentrations found in human serum induces aneugenic effects in endothelial cells. Mutat Res 751:27-33.
Ribeiro-Varandas E, Pereira HS, Monteiro S, Neves E, Brito L, Ferreira RB, Viegas W, Delgado M: Bisphenol A Disrupts Transcription and Decreases Viability in Aging Vascular Endothelial Cells. Int J Mol Sci 2014, in press.
Riganti C, Doublier S, Viarisio D, Miraglia E, Pescarmona G, Ghigo D, et al. 2009. Artemisinin induces doxorubicin resistance in human colon cancer cells via calcium-dependent activation of hif-1alpha and p-glycoprotein overexpression. Br J Pharmacol 156:1054-1066.

Robert V, Mouille B, Mayeur C, Michaud M, Blachier F. 2001. Effects of the garlic compound diallyl disulfide on the metabolism, adherence and cell cycle of ht-29 colon carcinoma cells: Evidence of sensitive and resistant sub-populations. Carcinogenesis 22:1155-1161.
Roeder E, Wiedenfeld H. 2013. Plants containing pyrrolizidine alkaloids used in the traditional indian medicine--including ayurveda. Pharmazie 68:83-92.
Rucker G, Schenkel EP, Manns D, Mayer R, Hausen BM, Heiden K. 1997. Allergenic sesquiterpene lactones from eupatorium cannabinum l. And kaunia rufescens (lund ex de candolle). Nat Toxins 5:223-227.
Schmidt GJ, Schilling EE. 2000. Phylogeny and biogeography of eupatorium (asteraceae: Eupatorieae) based on nuclear its sequence data. Am J Bot 87:716-726.
Shaulian E, Karin M. 2002. Ap-1 as a regulator of cell life and death. Nat Cell Biol 4:E131-136.
Sulsen VP, Cazorla SI, Frank FM, Redko FC, Anesini CA, Coussio JD, et al. 2007. Trypanocidal and leishmanicidal activities of flavonoids from argentine medicinal plants. Am J Trop Med Hyg 77:654-659.
Turck N, Richert S, Gendry P, Stutzmann J, Kedinger M, Leize E, et al. 2004. Proteomic analysis of nuclear proteins from proliferative and differentiated human colonic intestinal epithelial cells. Proteomics 4:93-105.
Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM. 2009. Bisphenol-a and the great divide: A review of controversies in the field of endocrine disruption. Endocrine Reviews 30:75-95.
Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgartten FJ, Schoenfelder G. 2010. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol a. Environ Health Perspect 118:1055-1070.
Woerdenbag HJ, Lemstra W, Malingre TM, Konings AW. 1989a. Enhanced cytostatic activity of the sesquiterpene lactone eupatoriopicrin by glutathione depletion. Br J Cancer 59:68-75.
Woerdenbag HJ, van der Linde JC, Kampinga HH, Malingre TM, Konings AW. 1989b. Induction of DNA damage in ehrlich ascites tumour cells by exposure to eupatoriopicrin. Biochem Pharmacol 38:2279-2283.
Xia Q, Zhao Y, Von Tungeln LS, Doerge DR, Lin G, Cai L, et al. 2013. Pyrrolizidine alkaloid-derived DNA adducts as a common biological biomarker of pyrrolizidine alkaloid-induced tumorigenicity. Chem Res Toxicol 26:1384-1396.
Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. 1993. P21 is a universal inhibitor of cyclin kinases. Nature 366:701-704.
Yu CY, Su KY, Lee PL, Jhan JY, Tsao PH, Chan DC, et al. 2013. Potential therapeutic role of hispidulin in gastric cancer through induction of apoptosis via nag-1 signaling. Evid Based Complement Alternat Med 2013:518301.
Zhang ML, Wu M, Zhang JJ, Irwin D, Gu YC, Shi QW. 2008. Chemical constituents of plants from the genus eupatorium. Chem Biodivers 5:40-55.

7 General Discussion

Presently the scientific community debates with a great controversy between the established TDI level for BPA (EFSA 2006, 2010), estimate intake levels based on migration values (WHO 2010) and biomonitoring studies on detected internal levels of unmetabolized BPA (Vandenberg et al. 2010). BPA effects have been extensively investigated in human cell lines associated with estrogenic receptors signalling pathways. Several lines of evidence established that this EDC promotes variable cellular responses frequently associated to cell type specificities. However the effects of BPA on human cells which are in continuous direct contact to this chemical *in vivo*, specifically digestive tract cells and vascular endothelial cells are mostly unknown. In this work the effects of two BPA concentrations, namely 10 ng/ml (44 nM), *i.e*. in the range detected in human blood at environmental exposures (Vandenberg et al. 2010) and 1 µg/ml (4.4 µM) associated to occupational exposure (He et al. 2009; Li et al. 2010) were evaluated in two cell types. Human Colon Adenocarcinona cell line (HT29) was selected as this type of cancer is one of the most common causes of death in industrialized countries (WHO 2014) and Human Umbilical Vein Endothelial Cells (HUVEC) was selected as representative of primary vascular endothelial cells tissues which undergo senescence in culture (Khaidakov et al. 2011). Both HT29 and HUVEC express the classical ERβ but are negative for ERα (Arai et al. 2000; Campbell-Thompson et al. 2001; Matthews et al. 2001; Toth et al. 2008). In relation to the membrane bound estrogen receptor GPR30, for which BPA has considerable high affinity (Thomas and Dong 2006), it is not expressed in HUVEC in standard culture conditions (Takada et al. 1997) ant it is demonstrated here its expression in HT29 cell line. Previously GPR30 expression in a ERβ-positive and ERα-negative cell line was correlated with BPA increased cell proliferation (Bouskine et al. 2009). However, we did not observe any BPA effect on cellular proliferation in either HT29 or HUVEC, indicating that BPA induced proliferation in GPR30-positive cell is dependent on cell type.

Interestingly, despite the deferential expression of GPR30, our results show that overall HUVEC were more susceptible to BPA exposure than HT29 cells. We

demonstrate for the first time that low-dose exposure to BPA increases the proportion of micronuclei formation in HUVEC, what is probably associated with spindle disruption, as previously reported for higher BPA concentrations in other cell lines (Nakagomi et al. 2001; Parry et al. 2002). HUVEC also show higher sensitivity to BPA-induced nucleolar disruption evidenced by altered nucleolar architecture and organization accompanied by variation on intranucleolar epigenetic marks H3K9me2 and H3K4me3, associated with transcriptional silencing and competence, respectively (Bartova et al. 2010; Hon et al. 2009). Although the global nuclear distribution pattern of these epigenetic marks is not affected by BPA exposure in both HT29 and HUVEC, induced modifications at specific genes cannot be excluded, particularly considering that BPA is known to affect the transcriptional expression of several genes involved in major cellular processes in different cell lines (Boehme et al. 2009; Bredhult et al. 2009; Buterin et al. 2006; Naciff et al. 2010). In fact, our transcriptional analysis also reveal HUVEC higher sensitivity to BPA since more pronounced effects on expression patterns of genes encoding for proteins involved in chromosome segregation as well as ribosome subunit biogenesis were observed. Additionally general transcription analysis based in the relative transcription of two LINE-1 related sequences shows a time and dose dependent effect of BPA on HUVEC whereas in HT29 cell line the response to BPA is lost with prolonged exposure.

The lower responsiveness of HT29 cells to BPA may be correlated with intrinsic characteristics of cancer cells which are described to be more insensitive to external stimulus (Hanahan and Weinberg 2011). Nevertheless it is important to refer that BPA-induced alterations in HT29 cells, namely transcription deregulation of LINE-1 related sequences and alteration of nucleolar morphology, are observed exclusively for the lower BPA concentration assayed. Hence our results suggest that BPA action in colon cancer cells may follow a nonmonotonic dose response (NMDR), what may be a common feature of natural hormones as well as EDCs (reviewed in Vandenberg et al. 2012). On the other hand, the results obtained from gene transcription and cell viability analysis show that continuous exposure to BPA interferes with HUVEC aging processes.. HUVEC are *in vitro* model for atherosclerosis pathogenesis (Khaidakov et al. 2011) and epidemiologic studies revealed a positive

correlation between BPA exposure and vascular diseases (Lind and Lind, 2011; Melzer et al., 2012; Melzer et al., 2010). Overall, our data indicate that BPA may play a role in atherosclerosis induction corroborating a potential impact on aging processes as previously suggested for mammary cells (Qin et al. 2012).

In relation to colon cancer cells it is important to take into consideration that digestive tract cells contact with ingested BPA prior to its conjugation what contributes to the overall cell exposure. Additionally the increase of extracellular β-glucuronidase levels in most solid tumors microenvironment (Tranoy-Opalinski et al. 2014) leads to a high level of uncertainty regarding BPA exposure. BPA potential interactions with chemotherapeutic drugs is an extremely poorly investigated issue, however in breast cancer cells it has been suggested that BPA is able to antagonize cytotoxic effects of distinct commonly used antineoplasic drugs including doxorubicin (DOX) (LaPensee et al. 2009). Interestingly, the present results show that in colon cancer cells BPA at a concentration derived from the TDI is able to interfere with DOX at therapeutic relevant level by antagonizing DOX-induced transcriptional effects on key genes involved in cancer biology. Surprisingly, these DOX-antagonistic effects of BPA are not observed for a 100-fold lower DOX concentration nor BPA *per se* induces major alterations on the transcription levels of the genes analyzed. Taken together these results emphasize the urge to address BPA interactions in the context of chemotherapy and raise important questions regarding BPA mode of action and consequences of the interactions on cancer cell fate.

In this work BPA interactions with *Eupatorium cannabinum* extract were also investigated showing that BPA aneugentic effects are increased by co-exposure. *Eupatorium cannabinum* is a medicinal plant that has long been utilized in alternative and/or complementary medicine (Jaric et al. 2007) including as an anticancer agent (Roeder and Wiedenfeld 2013) however, the cellular effects of its extracts were never assessed. The results obtained demonstrate a potent cytotoxic activity of *E. cannabinum* against colon cancer cells. Additionally, *E. cannabinum* extract also show synergistic effects with DOX in the induction of HT29 cell death supporting its potential use for cancer alternative or complementary therapeutic strategies.

Altogether the present work substantiates increasing concerns regarding the potential adverse effects of BPA low-dose exposures on human health. This is evident as we clearly established the potential genotoxic aneugenic effect of this chemical in vascular cells and supported previous epidemiologic correlations between circulating BPA levels and age-related pathologies (Lind and Lind 2011; Melzer et al. 2010; Melzer et al. 2012). Additionally we have also demonstrated the importance of addressing the effects of BPA drug-interaction, particularly in the context of chemotherapy and chemoresistance, and raise the prospect that BPA effects may be potentiated by interaction with other compounds.

8 References to Introduction and General Discussion

Alonso-Magdalena P, Ropero AB, Soriano S, Garcia-Arevalo M, Ripoll C, Fuentes E, et al. 2012. Bisphenol-a acts as a potent estrogen via non-classical estrogen triggered pathways. Mol Cell Endocrinol 355:201-207.

Andersson H, Brittebo E. 2012. Proangiogenic effects of environmentally relevant levels of bisphenol a in human primary endothelial cells. Arch Toxicol 86:465-474.

Arai N, Strom A, Rafter JJ, Gustafsson JA. 2000. Estrogen receptor beta mRNA in colon cancer cells: Growth effects of estrogen and genistein. Biochemical and Biophysical Research Communications 270:425-431.

Bartova E, Horakova AH, Uhlirova R, Raska I, Galiova G, Orlova D, et al. 2010. Structure and epigenetics of nucleoli in comparison with non-nucleolar compartments. J Histochem Cytochem 58:391-403.

Boehme K, Simon S, Mueller SO. 2009. Gene expression profiling in ishikawa cells: A fingerprint for estrogen active compounds. Toxicology and Applied Pharmacology 236:85-96.

Bolli A, Galluzzo P, Ascenzi P, Del Pozzo G, Manco I, Vietri MT, et al. 2008. Laccase treatment impairs bisphenol a-induced cancer cell proliferation affecting estrogen receptor alpha-dependent rapid signals. IUBMB Life 60:843-852.

Bolli A, Bulzomi P, Galluzzo P, Acconcia F, Marino M. 2010. Bisphenol A impairs estradiol-induced protective effects against dld-1 colon cancer cell growth. IUBMB Life 62:684-687.

Bouskine A, Nebout M, Brucker-Davis F, Benahmed M, Fenichel P. 2009. Low doses of bisphenol A promote human seminoma cell proliferation by activating PKA and PKG via a membrane G-protein-coupled estrogen receptor. Environ Health Perspect 117:1053-1058.

Bredhult C, Sahlin L, Olovsson M. 2009. Gene expression analysis of human endometrial endothelial cells exposed to bisphenol A. Reprod Toxicol 28:18-25.

Buterin T, Koch C, Naegeli H. 2006. Convergent transcriptional profiles induced by endogenous estrogen and distinct xenoestrogens in breast cancer cells. Carcinogenesis 27:1567-1578.

Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL. 2008. Exposure of the U.S. Population to bisphenol A and 4-tertiary-octylphenol: 2003-2004. Environ Health Perspect 116:39-44.

Campbell-Thompson M, Lynch IJ, Bhardwaj B. 2001. Expression of estrogen receptor (ER) subtypes and ER beta isoforms in colon cancer. Cancer Research 61:632-640.

Can A, Semiz O, Cinar O. 2005. Bisphenol-A induces cell cycle delay and alters centrosome and spindle microtubular organization in oocytes during meiosis. Mol Hum Reprod 11:389-396.

Dairkee SH, Luciani-Torres MG, Moore DH, Goodson WH, 3rd. 2013. Bisphenol-A-induced inactivation of the p53 axis underlying deregulation of proliferation kinetics, and cell death in non-malignant human breast epithelial cells. Carcinogenesis 34:703-712.

Debacq-Chainiaux F, Pascal T, Boilan E, Bastin C, Bauwens E, Toussaint O. 2008. Screening of senescence-associated genes with specific DNA array reveals the role of igfbp-3 in premature senescence of human diploid fibroblasts. Free Radic Biol Med 44:1817-1832.

Diel P, Olff S, Schmidt S, Michna H. 2002. Effects of the environmental estrogens bisphenol A, o,p'-ddt, p-tert-octylphenol and coumestrol on apoptosis induction, cell proliferation and the expression of estrogen sensitive molecular parameters in the human breast cancer cell line MCF-7. J Steroid Biochem Mol Biol 80:61-70.

Doerge DR, Twaddle NC, Vanlandingham M, Fisher JW. 2010a. Pharmacokinetics of bisphenol A in neonatal and adult sprague-dawley rats. Toxicol Appl Pharmacol 247:158-165.
Doerge DR, Twaddle NC, Woodling KA, Fisher JW. 2010b. Pharmacokinetics of bisphenol A in neonatal and adult rhesus monkeys. Toxicol Appl Pharmacol 248:1-11.
Doerge DR, Twaddle NC, Vanlandingham M, Fisher JW. 2011. Pharmacokinetics of bisphenol A in neonatal and adult cd-1 mice: Inter-species comparisons with sprague-dawley rats and rhesus monkeys. Toxicol Lett 207:298-305.
Doherty LF, Bromer JG, Zhou Y, Aldad TS, Taylor HS. 2010. In utero exposure to diethylstilbestrol (des) or bisphenol-A (bpa) increases ezh2 expression in the mammary gland: An epigenetic mechanism linking endocrine disruptors to breast cancer. Horm Cancer 1:146-155.
Dolinoy DC, Huang D, Jirtle RL. 2007. Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. Proceedings of the National Academy of Sciences of the United States of America 104:13056-13061.
Dong S, Terasaka S, Kiyama R. 2010. Bisphenol A induces a rapid activation of erk1/2 through GPR30 in human breast cancer cells. Environ Pollut 159:212-218.
Dumont P, Chainiaux F, Eliaers F, Petropoulou C, Remacle J, Koch-Brandt C, et al. 2002. Overexpression of apolipoprotein J in human fibroblasts protects against cytotoxicity and premature senescence induced by ethanol and tert-butylhydroperoxide. Cell Stress Chaperones 7:23-35.
Edginton AN, Ritter L. 2009. Predicting plasma concentrations of bisphenol A in children younger than 2 years of age after typical feeding schedules, using a physiologically based toxicokinetic model. Environ Health Perspect 117:645-652.
EFSA European Food Safety Authority 2006. Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food on a request from the commition related to 2.2 - bis(4-hydroxyphenyl) propane (bisphenol A).The EFSA Journal 428: 1-75. Available at: “http://www.efsa.europa.eu/fr/efsajournal/pub/428.htm”. [accessed February 2014].
EFSA European Food Safety Authority 2008. Toxicokinetics of bisphenol A - scientific opinion of the panel on food additives, flavourings, processing aids and materials in contact with food. The EFSA Journal 759:1-10 Available: at “http://www.efsa.europa.eu/fr/efsajournal/pub/759.htm”. [accessed February 2014].
EFSA European Food Safety Authority 2010. Scientific opinion on bisphenol A: Evaluation on a study investigating its neurodevelopmental toxicity, review of recent scientific literature on its toxicity and advice on the danish risk assessment of bisphenol A. The EFSA Journal (2010); 8(9):1829. Available at: “http://www.efsa.europa.eu/en/efsajournal/pub/1829.htm.” [accessed February 2014].
EFSA European Food Safety Authority 2014. Bisphenol A. Available at: "http://www.efsa.europa.eu/en/topics/topic/bisphenol.htm". [accessed february 2014].
Ehrlich S, Williams PL, Missmer SA, Flaws JA, Berry KF, Calafat AM, et al. 2012. Urinary bisphenol A concentrations and implantation failure among women undergoing in vitro fertilization. Environ Health Perspect 120:978-983.
EPA US. Environmental Protection Agency 1993. Integrated Risk Information System (IRIS) - Bisphenol A. Available at: "http://www.epa.gov/iris/subst/0356.htm". [accessed February 2014]
EU European Commission 2002. Opinion of the Scientific Committee on Food on Bisphenol A. Available at: "http://ec.europa.eu/food/fs/sc/scf/out128_en.pdf". [accessed February 2014].
EU European Commission 2004. Commission directive 2004/19/EC. Official Journal L 71, 10 March 2004, p. 8-21 Available at : "http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:071:0008:0021:EN:PDF". [accessed February 2014].

EU European Commission 2011. Commission directive 2011/8/EU. Official Journal L 26, 28 January 2011, 11-14 Available at: "http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:026:0011:0014:EN:PDF". [accessed February 2014].
Fernandez SV, Huang Y, Snider KE, Zhou Y, Pogash TJ, Russo J. 2012. Expression and DNA methylation changes in human breast epithelial cells after bisphenol A exposure. Int J Oncol 41:369-377.
Fisher JW, Twaddle NC, Vanlandingham M, Doerge DR. 2011. Pharmacokinetic modeling: Prediction and evaluation of route dependent dosimetry of bisphenol A in monkeys with extrapolation to humans. Toxicol Appl Pharmacol 257:122-136.
Fregert S, Rorsman H. 1962. Hypersensitivity to epoxy resins with reference to the role played by bisphenol A. J Invest Dermatol 39:471-472.
Gasnier C, Laurant C, Decroix-Laporte C, Mesnage R, Clair E, Travert C, et al. 2011. Defined plant extracts can protect human cells against combined xenobiotic effects. J Occup Med Toxicol 6:3.
Gaul LE. 1960. Sensitivity to bisphenol a. Arch Dermatol 82:1003.
Geens T, Goeyens L, Covaci A. 2011. Are potential sources for human exposure to bisphenol-A overlooked? Int J Hyg Environ Health 214:339-347.
Geens T, Aerts D, Berthot C, Bourguignon JP, Goeyens L, Lecomte P, et al. 2012. A review of dietary and non-dietary exposure to bisphenol-A. Food Chem Toxicol 50:3725-3740.
George O, Bryant BK, Chinnasamy R, Corona C, Arterburn JB, Shuster CB. 2008. Bisphenol A directly targets tubulin to disrupt spindle organization in embryonic and somatic cells. ACS Chem Biol 3:167-179.
Ginsberg G, Rice DC. 2009. Does rapid metabolism ensure negligible risk from bisphenol A? Environ Health Perspect 117:1639-1643.
Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: The next generation. Cell 144:646-674.
He Y, Miao M, Wu C, Yuan W, Gao E, Zhou Z, et al. 2009. Occupational exposure levels of bisphenol A among chinese workers. J Occup Health 51:432-436.
Hengstler JG, Foth H, Gebel T, Kramer PJ, Lilienblum W, Schweinfurth H, et al. 2011. Critical evaluation of key evidence on the human health hazards of exposure to bisphenol A. Crit Rev Toxicol 41:263-291.
Hon G, Wang W, Ren B. 2009. Discovery and annotation of functional chromatin signatures in the human genome. PLoS Comput Biol 5:e1000566.
Hugo ER, Brandebourg TD, Woo JG, Loftus J, Alexander JW, Ben-Jonathan N. 2008. Bisphenol A at environmentally relevant doses inhibits adiponectin release from human adipose tissue explants and adipocytes. Environ Health Perspect 116:1642-1647.
Hwang ES, Yoon G, Kang HT. 2009. A comparative analysis of the cell biology of senescence and aging. Cell Mol Life Sci 66:2503-2524.
Hwang KA, Kang NH, Yi BR, Lee HR, Park MA, Choi KC. 2013a. Genistein, a soy phytoestrogen, prevents the growth of bg-1 ovarian cancer cells induced by 17beta-estradiol or bisphenol A via the inhibition of cell cycle progression. Int J Oncol 42:733-740.
Hwang KA, Park MA, Kang NH, Yi BR, Hyun SH, Jeung EB, et al. 2013b. Anticancer effect of genistein on bg-1 ovarian cancer growth induced by 17 beta-estradiol or bisphenol A via the suppression of the crosstalk between estrogen receptor alpha and insulin-like growth factor-1 receptor signaling pathways. Toxicol Appl Pharmacol 272:637-646.
Iso T, Watanabe T, Iwamoto T, Shimamoto A, Furuichi Y. 2006. DNA damage caused by bisphenol A and estradiol through estrogenic activity. Biol Pharm Bull 29:206-210.
Jaric S, Popovic Z, Macukanovic-Jocic M, Djurdjevic L, Mijatovic M, Karadzic B, et al. 2007. An ethnobotanical study on the usage of wild medicinal herbs from kopaonik mountain (Central Serbia). J Ethnopharmacol 111:160-175.
Johnson GE, Parry EM. 2008. Mechanistic investigations of low dose exposures to the genotoxic compounds bisphenol-A and rotenone. Mutat Res 651:56-63.

Khaidakov M, Wang X, Mehta JL. 2011. Potential involvement of lox-1 in functional consequences of endothelial senescence. PLoS One 6:e20964.
Kim K, Son TG, Kim SJ, Kim HS, Kim TS, Han SY, et al. 2007. Suppressive effects of bisphenol A on the proliferation of neural progenitor cells. J Toxicol Environ Health A 70:1288-1295.
Knaak JB, Sullivan LJ. 1966. Metabolism of bisphenol A in the rat. Toxicol Appl Pharmacol 8:175-184.
Krishnan AV, Stathis P, Permuth SF, Tokes L, Feldman D. 1993. Bisphenol-A: An estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 132:2279-2286.
Lagos-Cabre R, Moreno RD. 2012. Contribution of environmental pollutants to male infertily: A working model of germ cell apoptosis induced by plasticizers. Biol Res 45:5-14.
Lakind JS, Naiman DQ. 2010. Daily intake of bisphenol A and potential sources of exposure: 2005-2006 national health and nutrition examination survey. J Expo Sci Environ Epidemiol 21:272-279.
LaPensee EW, Tuttle TR, Fox SR, Ben-Jonathan N. 2009. Bisphenol A at low nanomolar doses confers chemoresistance in estrogen receptor-alpha-positive and -negative breast cancer cells. Environ Health Perspect 117:175-180.
LaPensee EW, LaPensee CR, Fox S, Schwemberger S, Afton S, Ben-Jonathan N. 2010. Bisphenol A and estradiol are equipotent in antagonizing cisplatin-induced cytotoxicity in breast cancer cells. Cancer Lett 290:167-173.
Lee YH, Kim GE, Cho HJ, Yu MK, Bhattarai G, Lee NH, et al. 2013. Aging of in vitro pulp illustrates change of inflammation and dentinogenesis. J Endod 39:340-345.
Li DK, Zhou Z, Qing D, He Y, Wu T, Miao M, et al. 2010. Occupational exposure to bisphenol-A (bpa) and the risk of self-reported male sexual dysfunction. Hum Reprod 25:519-527.
Li DK, Zhou Z, Miao M, He Y, Wang J, Ferber J, et al. 2011. Urine bisphenol-A (bpa) level in relation to semen quality. Fertil Steril 95:625-630 e621-624.
Lind PM, Lind L. 2011. Circulating levels of bisphenol A and phthalates are related to carotid atherosclerosis in the elderly. Atherosclerosis 218:207-213.
MRC Merchant Research and Consulting Ltd 2014. World BPA Production Grew by Over 372,000 Tonnes in 2012. Available at: “http://mcgroup.Co.Uk/news/20131108/bpa-production-grew-372000-tonnes.html”. [accessed February 2014].
Matsushima A, Kakuta Y, Teramoto T, Koshiba T, Liu X, Okada H, et al. 2007. Structural evidence for endocrine disruptor bisphenol A binding to human nuclear receptor ERR gamma. J Biochem 142:517-524.
Matthews JB, Twomey K, Zacharewski TR. 2001. In vitro and in vivo interactions of bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptors alpha and beta. Chem Res Toxicol 14:149-157.
Melzer D, Rice NE, Lewis C, Henley WE, Galloway TS. 2010. Association of urinary bisphenol A concentration with heart disease: Evidence from nhanes 2003/06. PLoS One 5:e8673.
Melzer D, Gates P, Osborne NJ, Henley WE, Cipelli R, Young A, et al. 2012. Urinary bisphenol A concentration and angiography-defined coronary artery stenosis. PLoS One 7:e43378.
Mielke H, Gundert-Remy U. 2009. Bisphenol A levels in blood depend on age and exposure. Toxicol Lett 190:32-40.
Mlynarcikova A, Macho L, Fickova M. 2013. Bisphenol A alone or in combination with estradiol modulates cell cycle- and apoptosis-related proteins and genes in MCF7 cells. Endocr Regul 47:189-199.
Morice L, Benaitreau D, Dieudonne MN, Morvan C, Serazin V, de Mazancourt P, et al. 2011. Antiproliferative and proapoptotic effects of bisphenol A on human trophoblastic jeg-3 cells. Reprod Toxicol 32:69-76.
Naciff JM, Khambatta ZS, Reichling TD, Carr GJ, Tiesman JP, Singleton DW, et al. 2010. The genomic response of ishikawa cells to bisphenol A exposure is dose- and time-dependent. Toxicology 270:137-149.

Nakagomi M, Suzuki E, Usumi K, Saitoh Y, Yoshimura S, Nagao T, et al. 2001. Effects of endocrine disrupting chemicals on the microtubule network in chinese hamster V79 cells in culture and in sertoli cells in rats. Teratog Carcinog Mutagen 21:453-462.

NTP National Toxicology Program, US Department of Health and Human Services 1982. Carcinogenesis bioassay of bisphenol A in F344 rat and B6C3f$_1$ mice (feed study). Technical Report series Nº215. Available at: "http://ntp.niehs.nih.gov/?objectid=0706194F-FF39-0F1B-74A83661261ABA96". [accessed February 2014].

NTP National Toxicology Program, US Department of Health and Human Services 2001. Endocrine Disruptors Low-Dose Peer Review Final Report. Available at: http://ntp.niehs.nih.gov/ntp/htdocs/liason/LowDosePeerFinalRpt.pdf. [accessed February 2014].

Parry EM, Parry JM, Corso C, Doherty A, Haddad F, Hermine TF, et al. 2002. Detection and characterization of mechanisms of action of aneugenic chemicals. Mutagenesis 17:509-521.

Pottenger LH, Domoradzki JY, Markham DA, Hansen SC, Cagen SZ, Waechter JM, Jr. 2000. The relative bioavailability and metabolism of bisphenol A in rats is dependent upon the route of administration. Toxicol Sci 54:3-18.

Ptak A, Wrobel A, Gregoraszczuk EL. 2011. Effect of bisphenol-A on the expression of selected genes involved in cell cycle and apoptosis in the ovcar-3 cell line. Toxicol Lett 202:30-35.

Pupo M, Pisano A, Lappano R, Santolla MF, De Francesco EM, Abonante S, et al. 2012. Bisphenol A induces gene expression changes and proliferative effects through GPER in breast cancer cells and cancer-associated fibroblasts. Environ Health Perspect 120:1177-1182.

Qin XY, Fukuda T, Yang L, Zaha H, Akanuma H, Zeng Q, et al. 2012. Effects of bisphenol A exposure on the proliferation and senescence of normal human mammary epithelial cells. Cancer Biol Ther 13:296-306.

Ricupito A, Del Pozzo G, Diano N, Grano V, Portaccio M, Marino M, et al. 2009. Effect of bisphenol A with or without enzyme treatment on the proliferation and viability of MCF-7 cells. Environ Int 35:21-26.

Rochester JR. 2013. Bisphenol A and human health: A review of the literature. Reprod Toxicol 42:132-155.

Roeder E, Wiedenfeld H. 2013. Plants containing pyrrolizidine alkaloids used in the traditional indian medicine--including ayurveda. Pharmazie 68:83-92.

Routledge EJ, White R, Parker MG, Sumpter JP. 2000. Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (er) alpha and erbeta. J Biol Chem 275:35986-35993.

Rudel RA, Gray JM, Engel CL, Rawsthorne TW, Dodson RE, Ackerman JM, et al. 2011. Food packaging and bisphenol A and bis(2-ethyhexyl) phthalate exposure: Findings from a dietary intervention. Environ Health Perspect 119:914-920.

Schonfelder G, Wittfoht W, Hopp H, Talsness CE, Paul M, Chahoud I. 2002. Parent bisphenol A accumulation in the human maternal-fetal-placental unit. Environmental Health Perspectives 110:A703-A707.

Sekizawa J. 2008. Low-dose effects of bisphenol A: A serious threat to human health? Journal of Toxicological Sciences 33:389-403.

Shanle EK, Xu W. 2010. Endocrine disrupting chemicals targeting estrogen receptor signaling: Identification and mechanisms of action. Chem Res Toxicol 24:6-19.

Shelton DN, Chang E, Whittier PS, Choi D, Funk WD. 1999. Microarray analysis of replicative senescence. Curr Biol 9:939-945.

Sieli PT, Jasarevic E, Warzak DA, Mao J, Ellersieck MR, Liao C, et al. 2011. Comparison of serum bisphenol A concentrations in mice exposed to bisphenol A through the diet versus oral bolus exposure. Environ Health Perspect 119:1260-1265.

Silver MK, O'Neill MS, Sowers MR, Park SK. 2011. Urinary bisphenol A and type-2 diabetes in U.S. Adults: Data from nhanes 2003-2008. PLoS One 6:e26868.

Singh S, Li SS. 2012. Epigenetic effects of environmental chemicals bisphenol A and phthalates. Int J Mol Sci 13:10143-10153.

Stahlhut RW, Welshons WV, Swan SH. 2009. Bisphenol A data in nhanes suggest longer than expected half-life, substantial nonfood exposure, or both. Environ Health Perspect 117:784-789.
Takada Y, Kato C, Kondo S, Korenaga R, Ando J. 1997. Cloning of cDNAs encoding G protein-coupled receptor expressed in human endothelial cells exposed to fluid shear stress. Biochem Biophys Res Commun 240:737-741.
Takayanagi S, Tokunaga T, Liu X, Okada H, Matsushima A, Shimohigashi Y. 2006. Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor gamma (ERRgamma) with high constitutive activity. Toxicol Lett 167:95-105.
Tayama S, Nakagawa Y, Tayama K. 2008. Genotoxic effects of environmental estrogen-like compounds in cho-k1 cells. Mutat Res 649:114-125.
Taylor JA, Vom Saal FS, Welshons WV, Drury B, Rottinghaus G, Hunt PA, et al. 2011. Similarity of bisphenol A pharmacokinetics in rhesus monkeys and mice: Relevance for human exposure. Environ Health Perspect 119:422-430.
Teeguarden JG, Waechter JM, Jr., Clewell HJ, 3rd, Covington TR, Barton HA. 2005. Evaluation of oral and intravenous route pharmacokinetics, plasma protein binding, and uterine tissue dose metrics of bisphenol A: A physiologically based pharmacokinetic approach. Toxicol Sci 85:823-838.
Teeguarden JG, Hanson-Drury S. 2013. A systematic review of bisphenol A "Low dose" Studies in the context of human exposure: A case for establishing standards for reporting "Low-dose" Effects of chemicals. Food Chem Toxicol 62:935-948.
Thomas P, Dong J. 2006. Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: A potential novel mechanism of endocrine disruption. J Steroid Biochem Mol Biol 102:175-179.
Tominaga T, Negishi T, Hirooka H, Miyachi A, Inoue A, Hayasaka I, et al. 2006. Toxicokinetics of bisphenol A in rats, monkeys and chimpanzees by the lC-MS/MS method. Toxicology 226:208-217.
Toth B, Saadat G, Geller A, Scholz C, Schulze S, Friese K, et al. 2008. Human umbilical vascular endothelial cells express estrogen receptor beta (ER beta) and progesterone receptor A (PR-A), but not ER alpha and PR-B. Histochemistry and Cell Biology 130:399-405.
Toussaint O, Medrano EE, von Zglinicki T. 2000. Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. Exp Gerontol 35:927-945.
Tranoy-Opalinski I, Legigan T, Barat R, Clarhaut J, Thomas M, Renoux B, et al. 2014. Beta-glucuronidase-responsive prodrugs for selective cancer chemotherapy: An update. Eur J Med Chem 74:302-313.
Tyl RW, Myers CB, Marr MC, Thomas BF, Keimowitz AR, Brine DR, et al. 2002. Three-generation reproductive toxicity study of dietary bisphenol A in cd sprague-dawley rats. Toxicol Sci 68:121-146.
Uchida K, Suzuki A, Kobayashi Y, Buchanan D, Sato T, Watanabe H, et al. 2002. Bisphenol-A administration during pregnancy results in fetal exposure in mice and monkeys. J Health Sci 48:579–582.
Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. 2007. Human exposure to bisphenol A (bpa). Reproductive Toxicology 24:139-177.
Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM. 2009. Bisphenol-A and the great divide: A review of controversies in the field of endocrine disruption. Endocrine Reviews 30:75-95.
Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgartten FJ, Schoenfelder G. 2010. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. Environ Health Perspect 118:1055-1070.
Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Jr., Lee DH, et al. 2012. Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. Endocr Rev 33:378-455.
Verma RJ, Sangai NP. 2009. The ameliorative effect of black tea extract and quercetin on bisphenol A-induced cytotoxicity. Acta Pol Pharm 66:41-44.

Völkel W, Colnot T, Csanady GA, Filser JG, Dekant W. 2002. Metabolism and kinetics of bisphenol A in humans at low doses following oral administration. Chem Res Toxicol 15:1281-1287.
Völkel W, Bittner N, Dekant W. 2005. Quantitation of bisphenol A and bisphenol A glucuronide in biological samples by high performance liquid chromatography-tandem mass spectrometry. Drug Metab Dispos 33:1748-1757.
Wang T, Lu J, Xu M, Xu Y, Li M, Liu Y, et al. 2013. Urinary bisphenol A concentration and thyroid function in chinese adults. Epidemiology 24:295-302.
Weng YI, Hsu PY, Liyanarachchi S, Liu J, Deatherage DE, Huang YW, et al. 2010. Epigenetic influences of low-dose bisphenol A in primary human breast epithelial cells. Toxicol Appl Pharmacol 248:111-121.
Wens B, De Boever P, Verbeke M, Hollanders K, Schoeters G. 2013. Cultured human peripheral blood mononuclear cells alter their gene expression when challenged with endocrine-disrupting chemicals. Toxicology 303:17-24.
Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenscheing C, et al. 2007. In vitro molecular mechanisms of bisphenol A action. Reproductive Toxicology 24:178-198.
WHO World Health Organization 2010. Joint FAO/WHO Expert Meeting to Review Toxicological and Health Aspects of Bisphenol A. Available at: "http://www.who.int/foodsafety/chem/chemicals/bisphenol_release/en/index.html". [accessed February 2014].
WHO World Health Organization 2014. Cancer. Fact sheet n°297. Available at: "http://www.who.int/mediacentre/factsheets/fs297/en/" [accessed February 2014].
Zhu H, Xiao X, Zheng J, Zheng S, Dong K, Yu Y. 2009. Growth-promoting effect of bisphenol A on neuroblastoma in vitro and in vivo. J Pediatr Surg 44:672-680.

9 List of abbreviations

AURKA ..... Aurora A kinase gene
BBE..... Bovine Brain Extract
bcl-xl ..... B-cell lymphoma-extra large gene
BPA..... Bisphenol A
BSA..... Bovine Serum Albumin
CDCA8..... Cell Division Cycle Associated 8 gene
cDNA ..... complementary DNA
Ct ..... Threshold cycles
Cy3 ..... Cyanine 3
DAPI ..... 4',6-diamidino-2-phenylindole
DES ..... Diethylstilbestrol
DFC ..... Dense Fibrillar Component
DNA ..... DeoxyriboNucleic Acid
DNase..... deoxyribonuclease
DOX ..... Doxorubicin
E2..... 17 β- estradiol or oestradiol
EC_{50} ..... Concentration for 50% of Maximal Effect
ECACC ..... European Collection of Cell Cultures
EcEE..... *Eupatorium cannabinum* ethanolic extract
EDC(s)..... Endocrine Disrupting Chemical(s)
EFSA ..... European Food Safety Authority
(US)EPA ..... (U.S.) Environmental Protection Agency
ERb..... Estrogen receptor beta
ERα..... Estrogen receptor alpha
FBS..... Fetal Bovine Serum
FC ..... Fibrillar Centre
FISH..... Fluorescent in situ Hybridazation
FITC..... Fluorescein isothiocyanate
g ..... Standard gravity
GAPDH ..... Glyceraldehyde 3-phosphate dehydrogenase gene
GC..... Granular Component
GlutaMAX ..... L-Glutamina
GPR30..... G protein-coupled receptor 30
H_2O_2..... Hydrogen peroxide
H3K4me3..... Histone H3 tri-methylated on lysine 4

H3K56ac ..... Histone H3 acetylated on lysine 56
H3K9ac ..... Histone H3 acetylated on lysine 9
HbA1c ..... Glycated hemoglobin
HD ..... High Dosages
HEPES ..... 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hrs or h, min, sec ..... hours, minutes, seconds
HT29 ..... Human Colon Adenocarcinona cell line HT29
HUVEC ..... Human Umbilical Vascular Endothelial Cells
IgG ..... Immunoglobulin G
Kg, g, mg, µg, ng, pg ..... Kilogram, gram, milligram, microgram, nanogram, picogram
l or L, ml, µl ..... litres, millilitres, microlitres
LD ..... Low Dosages
LOAEL ..... Lowest Observable Adverse Effect Level
log ..... logarithm
M, mM, µM, nM. pM ..... Molar, miliMolar, microMolar, nanoMolar, picoMolar
miRNA ..... microRNA
mRNA ..... messenger RNA
NHANES ..... National Health and Nutrition Examination Survey
NMDR ..... Nonmonotonic Dose Responses
NOAEL ..... No Observable Effect Level
NOR ..... Nucleolar Organizing Regions
NTP ..... National Toxicology Program
ºC ..... degree Celsius
PBS ..... Phosphate Buffered Saline
PBST ..... Phosphate Buffered Saline Tween
PVDF ..... Polyvinylidene difluoride
qRT-PCR ..... quantitative Real Time Polymerase Chain Reaction
rDNA ..... ribossomal DNA
RfD ..... Oral Reference Dosage
RNA ..... RiboNucleic Acid
RNase ..... Ribonuclease
rRNA ..... ribosomal RNA
SDS ..... Sodium Dodecyl Sulphate
SGOL2 ..... Shugoshin-like 2 gene
TDI ..... Tolerable Daily Intake
Tm ..... melting Temperature
TUNEL ..... Terminal deoxynucleotidyl transferase dUTP nick end labeling

v/v, w/v..... Volume per Volume, weight per volume

WHO..... World Health Organization

α- tubulin..... alpha tubulin

β-actin..... Beta actin

γ-tubulin..... gama Tubulin

Δ..... Difference

μm, nm..... micrometer, nanometre

μmol, nmol..... micromole, nanomole

10 Acknowledgments/Agradecimentos

Agora, no final desta jornada maravilhosa, com todos os seus altos e baixos, não posso deixar de agradecer a todos os que fizeram parte dela e a tornaram possível, quer por estarem presentes no dia-a-dia, quer por viverem no meu coração.

Em primeiríssimo lugar tenho de agradecer às minhas grandes orientadoras, Professora Wanda Viegas e Doutora Margarida Delgado, por toda a dedicação, empenho, paciência, sabedoria e principalmente por toda a amizade ao longo destes anos.
Chefinha foi uma honra e acima de tudo um grande prazer trabalhar contigo! Se soubéssemos o que nos esperava! Faria tudo de novo! Foi uma grande aventura e uma aprendizagem fantástica. És e sempre serás uma pessoa muito especial para mim!

A todos os colegas e amigos do ISA.
Claro que não posso deixar de agradecer primeiro ao Bentolas!!! E agradeço por tantas coisas que seria uma nova tese... Ele sabe... Muito resumidamente tenho de agradecer pela amizade destes últimos 7 anos, sim já la vão 7 anos, por todo o apoio e compreensão que sempre me deu, pela cumplicidade, pelos disparates, pelos jantares e almoços, pelas canetas e lapiseiras... Enfim por ser meu amigo! Um grande amigo!!!
À Verynice que apareceu a meio desta jornada e foi uma grande alegria...
Aos companheiros de brainstorm Sofia, Filipe, Ana Costa, Ana Caperta, Neves, Manela e Leonor.
Aos coleguinhas de bancada Gusta, Diana, Rois, Carjé, Teresa, Ana Luisa e Rocheta.
Agradeço também á Elsa e á Carla pelo apoio nas “nossas meninas”.
Por fim á Menina e á Xaninha por todos os momentos bem passados.

À Raquel, Sofia, Diana e Ana agradeço de uma forma muito especial, por viverem no meu coração há tantos anos, por serem a minha família de alma e escolha, por terem estado “lá” nos melhores e piores momentos e por provarem que a amizade é o sentimento mais puro e acolhedor do mundo...mesmo longe estarão sempre comigo e sei que estarei sempre com elas.E ao Ricardo claroooo…

Ao Miguel que me acompanhou nesta jornada, me apoiou, incentivou, “levantou” nos momentos mais sombrios e acima de tudo comemorou a meu lado todas as vitórias. Obrigada maridão!

Aos meus pais, sem eles nunca teria chegado aqui! Obrigada por me darem coragem, dedicação e amor. Isto é por vocês!

À minha família, tia Ju, Mim, Tati, tio Eurico e Armando. Obrigada por tudos os momentos de união e felicidade ao longo dos anos e por fazerem esta família ser tão especial… São o meu porto de abrigo. A “presidente do CDM”. Á minha avó que estará sempre comigo...

Para terminar agradeço á minha filha, Maria Catarina, que se formou, cresceu, nasceu, deu os primeiros passos e declamou as primeiras palavras no decorrer desta jornada. A ela que transformou os momentos mais tristes em pura felicidade só com um sorriso...que me ensinou a ver “o outro lado”...que me mostrou o amor incondicional e que me deu forças quando pensava que as tinha perdido... A minha Lili... Tornaste o meu mundo mais doce meu amor pequenino!

Por fim agradeço á Fundação para a Ciência e Tecnologia pela bolsa de Doutoramento que me foi atribuida e me permitiu desenvolver este trabalho bem como aos projetos PTDC/AAC-AMB/103968/2008 and PEst-OE/AGR/UI0240/2011.